Lung infection with porcine reproductive and respiratory syndrome virus in naive and vaccinated pigs

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Table of contents

List of abbreviations

ADEI	antibody-dependent enhancement of infectivity
ANOVA	analysis of variance
BAL	broncho-alveolar lavage
CD	cluster of differentiation
CDCD	caesarean-derived colostrum-deprived
CSFV	classical swine fever virus
DABCO	1,4-diazobicyclo-2.2.2-octane
E	envelope
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FL	fluorescence
FSC	forward light scattering
GP	glycoprotein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
	interleukine
IN	intranasal
IPMA	immunoperoxidase monolayer assay
IT	intratracheal
kD	kilodalton
LAL	Limulus Amoebocyte Lysate
LBP	LPS-binding protein
LPS	lipopolysaccharide
LSD	least significant difference
M	matrix
MAb	monoclonal antibody
MW	molecular weight
mRNA	messenger RNA
N	nucleocapsid
ORF	open reading frame

P	protein
PAMs	pulmonary alveolar macrophages
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC	post challenge
PCR	polymerase chain reaction
PCV	porcine circovirus
PI	post inoculation
PRCV	porcine respiratory coronavirus
PRV	pseudorabies virus
PRRS	porcine reproductive and respiratory syndrome
PRRSV	porcine reproductive and respiratory syndrome virus
	porcien reproductief en respiratoir syndroom virus
SIV	swine influenza virus
SPF	specific pathogen-free
SSC	sideward light scattering
$TCID_{50}$	50% tissue culture infectious dose
TGEV	transmissible gastroenteritis virus
TLR4	Toll-like receptor 4
TNF-α	tumour necrosis factor-alpha
	tumor necrosis factor-alfa
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
U	unit

1.

INTRODUCTION

1.1. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS WITH EMPHASIS ON PATHOGENESIS, CLINICAL DISEASE AND VACCINATION

1.2. LIPOPOLYSACCHARIDES AND ROLE IN RESPIRATORY DISEASE

1. 1. Porcine reproductive and respiratory syndrome virus with emphasis on pathogenesis, clinical disease and vaccination

1.1.1. Introduction

In 1987, a new disease characterized by reproductive failure in breeding pigs and respiratory disease problems in nursery and fattening pigs, was observed in the USA and Canada (Keffaber, 1989; Hill, 1990). Three years later, a similar syndrome was reported in Germany (Ohlinger et al., 1991). Subsequently, the disease spread rapidly through the major swine-producing areas of Western Europe. In 1991, the causative agent of the disease was isolated and identified as Lelystad virus (Terpstra et al., 1991; Wensvoort et al., 1991). A similar virus was subsequently isolated in the USA (Collins et al., 1992). A wide variety of names have been designated to the disease including mystery swine disease, swine infertility and respiratory syndrome, porcine epidemic abortion and respiratory syndrome, and blue-eared pig disease. Since 1992, "porcine reproductive and respiratory syndrome (PRRS)" is the internationally recognized name applied to the syndrome (Collins et al., 1992) and the causative agent is named "porcine reproductive and respiratory syndrome virus (PRRSV)".

The first introduction of PRRSV in the susceptible swine population was characterized by acute outbreaks of late-term reproductive failure in breeding pigs and increased pre-weaning mortality and respiratory disease problems in nursery and fattening pigs. At present, the virus is enzootic in the swine population and its exact role in reproductive and respiratory disease problems is still under debate.

1.1.2. The virus

PRRSV is a small enveloped positive-strand RNA virus that belongs to the *Arteriviridae* family together with equine arteritis virus, lactate dehydrogenase-elevating virus of mice, and simian hemorrhagic fever virus (Cavanagh, 1997). PRRSV virions are spherical with a diameter ranging from 45 to 65 nm for European isolates (Ohlinger et al., 1991; Wensvoort et al., 1991) and from 48 to 83 nm for North American isolates (Benfield et al., 1992; Dea et al., 1992). The structure is given in Figure 1.

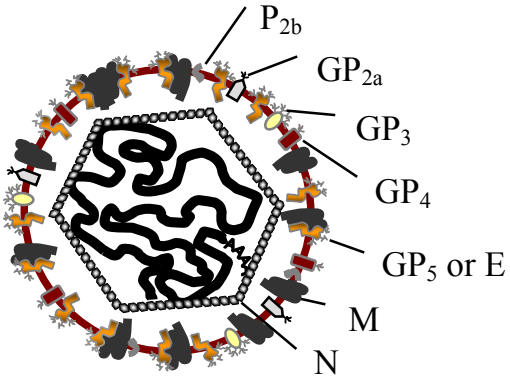

Fig. 1. Schematic representation of a PRRSV virion.

The inner part of the virion consists of an isometric nucleocapsid, 25 to 35 nm in diameter, composed by the single-strand, positive-sense RNA (15 kilobase pairs) and the 15-kD nucleocapsid (N) protein. The nucleocapsid is surrounded by a lipid bilayer, the envelope, which contains six structural proteins: P_{2b}, GP_{2a}, GP_3, GP_4, GP_5, and M. The 18- to 19-kD major non-N-glycosylated matrix (M) protein forms disulfide-linked heterodimers with the 25-kD major glycoprotein 5 (GP_5), also called the envelope (E) protein. Recently, a 10-kD 2b protein (P_{2b}) was identified (Wu et al., 2001). The 29- to 30-kD glycoprotein 2a (GP_{2a}) and the 31- to 35-kD glycoprotein 4 (GP_4) of PRRSV were identified as minor glycoproteins of the virion. Conflicting data exist about the presence of a 45- to 50-kD glycoprotein 3 (GP_3) in the envelope, since it has been demonstrated in Lelystad virus particles (van Nieuwstadt et al., 1996), but not in the Canadian IAF-klop isolate (Mardassi et al., 1998).

PRRSV is stable at –70 and –20°C. At higher temperatures, the half-life of PRRSV is 140 hours at 4°C, 20 hours at 21°C, 3 hours at 37°C and 6 minutes at 56°C (Benfield et al., 1992; Bloemraad et al., 1994).

PRRSV replicates only in a limited number of cell types. Of many swine cell types, only pulmonary alveolar macrophages (PAMs) and aged blood monocytes support a productive replication of PRRSV *in vitro*. Despite the fact that PAMs are the most sensitive cell type for PRRSV, these cells show some restriction to a PRRSV infection when they are freshly isolated. The susceptibility clearly increases after one-day cultivation, suggesting that the state of macrophage differentiation plays an important role in determining their susceptibility to PRRSV (Duan et al., 1997b). PRRSV can also

be cultivated in three established non-porcine cell lines: MARC-145 (Kim et al., 1993) and CL-2621 cells (Benfield et al., 1992), both originating from the embryonic monkey kidney cell line MA-104, and CRL-11171 cells (Meng et al., 1996). It has been reported by several researchers that the susceptibility of the different cell types differs among strains. Bautista et al. (1993b) demonstrated that, when using PAMs and CL-2621 cells, nearly one third of PRRSV isolates grown in one cell type failed to grow in the other one. Pol et al. (1997b) reported that Lelystad virus replicated very fast and efficiently in PAMs, while North American strains preferably replicated in CL-2621 cells.

PRRSV entry in macrophages occurs via receptor-mediated endocytosis and two PRRSV receptors were identified on PAMs (Duan et al., 1998b; Delputte et al., 2002). One PRRSV receptor was identified by generation of two monoclonal antibodies (MAbs), which were able to block PRRSV infection (Duan et al., 1998a). A protein with a molecular weight of approximately 210-kD was immuno-precipitated by these MAbs. This protein was identified as the porcine homologue of the mouse and the human sialoadhesin (Vanderheijden et al., 2002). Delputte et al. (2002) showed that addition of heparin or heparan sulfate to the virus, or heparinase treatment of PAMs, clearly reduced PRRSV infection, indicating that cell surface heparan sulfate glycosaminoglycans are a PRRSV receptor. Following binding to the cell receptors, virus particles become internalized by a microfilament-dependent process through small clathrin-like coated vesicles. During this stage, an acidic pH is required to trigger fusion between the viral envelope and the endosomal membrane allowing the viral nucleocapsid to enter the cytoplasm for replication (Nauwynck et al., 1999). Viral antigens are detected within the cytoplasm of PAMs as soon as 6 hours after inoculation. From this time on, the nucleocapsid buds at the smooth endoplasmatic reticulum (Pol et al., 1992). Virions seem to mature between the endoplasmatic reticulum and the Golgi apparatus, since budding and accumulation of enveloped virus particles can only be observed between these organels (Dea et al., 1995). One replication cycle of PRRSV takes about 9 to 12 hours (Pol, 1992). Cytopathic effect is visible after 24 hours (Paton et al., 1992; Bloemraad et al., 1994). The exact mechanism of cell death is unknown, but is likely to be through apoptosis. The GP_5 protein of the virus has been shown to induce apoptosis *in vitro* (Suárez et al., 1996a).

1.1.3. Antigenic and genetic differences

PRRSV isolates are classified into two distinct serotypes, namely the European and the North American serotype (Wensvoort et al., 1992; Bautista et al., 1993a). GP_5 is the most variable structural protein, with only 51 to 55% amino acid identity between European and North American isolates, whereas the M and 2b proteins are the most conserved structural proteins, with 78 to 81% and 74% amino acid identity, respectively (Meng et al., 1994; Kapur et al., 1996; Wu et al., 2001).

Nucleotide sequencing of all genes of European and North American isolates confirmed the existence of two subtypes. The Lelystad strain is, on the average, 35% divergent from North American isolates (Kapur et al., 1996). European and North American PRRSV isolates are genetically seen two distinct clusters of the same ancestor virus. It has been speculated that European and North American PRRSV isolates may have evolved from a lactate dehydrogenase-elevating virus-like ancestor (Plagemann, 1996; Nelsen et al., 1999). The large differences in genomic sequence indicate that the two viruses diverged before clinical disease was described in the late 1980s (Magar et al., 1995; Kapur et al., 1996). However, the almost simultaneous emergence of the syndrome on the two continents makes the theory of divergent evolution difficult to believe. A high degree of genetic diversity has been demonstrated within North American isolates (Magar et al., 1995; Kapur et al., 1996; Pirzadeh et al., 1998). Early reports suggested that European isolates exhibit a low degree of variability (Suárez et al., 1996b; Le Gall et al., 1998). However, more recently it was shown that isolates from Great Britain, Russia, Czech Republic, Denmark, Italy, Austria, Poland, and Lithunia are 9 to 18% divergent from the prototype Lelystad virus (Drew et al., 1997; Andreyev et al., 2000; Indik et al., 2000; Forsberg et al., 2001; Bignotti et al., 2002; Forsberg et al., 2002; Schmoll et al., 2002; Stadejek et al., 2002).

1.1.4. Epidemiology

PRRS was first observed in the USA in 1987 (Keffaber, 1989; Hill, 1990). There is, however, evidence that PRRSV has entered the domestic swine population several years before the clinical disease became visible. Swine sera collected in the mid-1980s in Canada, Korea, Japan, and Eastern Germany tested positive for PRRSV-specific antibodies (Kramer et al., 1993; Shin et al., 1993; Murakami, 1994; Carman et al., 1995; Dewey, 2000; Ohlinger et al., 2000).

PRRSV is highly infectious for pigs of all age. The virus is transmitted by direct pig-to-pig contact, by fomites (boots and coveralls) and via semen (Yaeger et al., 1993; Swenson et al., 1994; Christopher-Hennings et al., 1995a,b; Albina, 1997; Otake et al., 2002b). There are a lot of conflicting data about airborne transmission of the virus. The rapid spread of PRRSV through Western Europe immediately after the first outbreak in Germany suggested an easy airborne transmission of the virus. It has been demonstrated that PRRSV can be transmitted via the air at distances of about 3 km (Albina, 1997). One study even mentioned an airborne transmission of the virus at a distance of 20 km (de Jong et al., 1991). Le Potier et al. (1997), on the other hand, observed that many farms (45%) located in a 500 m radius around PRRSV outbreaks became infected, whereas only few (2%) became infected in the zone 1 to 2 km from an outbreak. Komijn et al. (1991) reported that airborne spread is generally enhanced during winter when temperature is low, humidity high, and when wind speed and ultra-violet light exposure are low. Some research groups were able to reproduce airborne transmission using two pig units, placed at a distance of 0.5-1 meter of each other and connected by pipes (Torremorell et al., 1997; Lager & Mengeling, 2000; Kristensen et al., 2002). Other research groups, however, did not succeed to achieve aerosol spread experimentally (Wills et al., 1994; Otake et al., 2002a). Thus, the role of aerosols in transmission of PRRSV is still under debate. Zimmerman et al. (1997) showed that some avian species may be involved in the epidemiology of PRRSV, since pigs, which were intranasally inoculated with PRRSV isolated from feces of mallard ducks, became viremic, seroconverted and transmitted the virus to sentinel pigs. Although mallard ducks may not be significant vectors in the field, it does indicate that birds can carry the virus and infect pigs. Rodents are not susceptible to PRRSV (Hooper et al., 1994). Otake et al. (2002c,d) reported that blood-borne transmission of PRRSV can be achieved by contaminated needles and by mosquitoes.

Once infected, pigs shed virus in nasal secretions, saliva, urine, and feces until at least 28 days after infection (Christianson et al., 1993; Yoon et al., 1993; Rossow et al., 1995). The low frequency of virus-positive fecal samples suggests that PRRSV is shed intermittently and at low levels in feces. Furthermore, it has been reported that PRRSV is rapidly inactivated in fecal slurry (Pirtle & Beran, 1996). Boars have been shown to shed infectious virus in their semen for up to 43 days (Swenson et al., 1994). Viral RNA in semen has been detected for up to 92 days (Christopher-Hennings et al., 1995a). It has been demonstrated that sows inoculated late in gestation shed PRRSV in mammary gland

secretions (Wagstrom et al., 2001). Shedding appeared to occur sporadically and at low levels. Senn et al. (1998) postulated that colostrum or milk can be a source of PRRSV.

The pattern of PRRSV infections on herd level has been studied by Houben et al. (1995). Most pigs are born from immune sows and thus have maternally derived antibodies during their first weeks of life. When an infection takes place, maternally derived immunity is replaced by active immunity. In the absence of infection, maternally derived antibodies do not persist beyond 10 to 11 weeks of age (Dee et al., 1993; Albina et al., 1994; Houben et al., 1995). The exact age of infection is variable between herds and even between litters in the same herd. A serological follow-up on 20 farrow-to-finish herds in Belgium showed that 40% of the pigs had been infected by 10 weeks of age, while 82% had seroconverted by 16 weeks of age and 92.5% had been infected by the end of the fattening period (Mateusen et al., 2002). Thus, many pigs become infected some time after entering the fattening units.

1.1.5. Pathogenesis

Pigs of all ages are susceptible to a PRRSV infection. Experimental infection can be achieved following intranasal, intratracheal, oronasal, oral, intramuscular, intrauterine, intravenous, or intraperitoneal inoculations (Wensvoort et al., 1991; Christianson et al., 1992; Collins et al., 1992; Christianson et al., 1993; Rossow et al., 1994; Swenson et al., 1994; Wills et al., 1994; Pol et al., 1997a; Van Reeth et al., 1999; Yoon et al., 1999). Under natural circumstances, the virus most frequently enters via the respiratory tract, but viraemia and dissemination throughout the body rapidly occur. The kinetics of a PRRSV infection in the lungs and lymphoid organs have been described in detail by Duan et al. (1997a) and Beyer et al. (2000). In both studies, inoculation with PRRSV rapidly resulted in viraemia and virus replication in several organs. From 2 to 4 days after inoculation onwards, it was possible to isolate PRRSV from lungs, tonsils, lymph nodes, thymus, spleen, and blood. The highest virus titres in tonsils and lungs were reached at 14 days after inoculation, whereas the highest virus titres in lymph nodes were reached at 3 days after inoculation. From tonsils, lymph nodes and thymus, virus isolation was possible up to 21 days after inoculation. From lungs, infectious virus was recovered until 35 days after inoculation. PRRSV-infected cells were mostly located in the alveolar spaces of the lungs and in germinal centers of lymphoid follicles in lymph nodes and spleen. Viraemia was detected until 21 to 28 days after inoculation. The latter finding

was confirmed by other researchers (Halbur et al., 1996; Wills et al., 1997). However, viraemia of up to 5-9 weeks duration has also been reported (Yoon et al., 1993; Bilodeau et al., 1994; Sur et al., 1997). During viraemia, the virus may be distributed to various organs. In boars, the virus may infect the reproductive tract and be shed in semen (Swenson et al., 1994; Christopher-Hennings et al., 1995a,b). In pregnant sows, PRRSV is able to cross the placenta. The efficiency by which PRRSV crosses the placenta depends on the stage of gestation. At early- and mid-gestation, transplacental infection is rarely observed (Christianson et al., 1993; Mengeling et al., 1994). However, during late gestation (93 days of gestation), transplacental infection occurs easily (Christianson et al., 1992). These differences may be explained by differences in placental permeability during gestation (Christianson et al., 1993). The virus has further been detected in nasal turbinates, kidneys, brains, liver, trachea, bone marrow, and choroid plexus (Pol et al., 1991; Rossow et al., 1994; Halbur et al., 1995; Rossow et al., 1995; Beyer et al., 2000).

Cells of the monocyte/macrophage lineage are the main target cells for PRRSV. The susceptibility of these cells to PRRSV varies, however, in different organs. Macrophages in lungs, tonsils, lymph nodes, and spleen are permissive to PRRSV infection (Duan et al., 1997a,b; Thanawongnuwech et al., 1997; Beyer et al., 2000), whereas virus infection was not detected in macrophages of liver, kidneys and heart, or in macrophage precursor cells such as blood mononuclear cells and bone marrow cells (Duan et al., 1997a,b). Virus replication has also been shown in microglial cells (Molitor et al., 1996). It is remarkable that only 2% of the alveolar macrophages, which are the main target cells *in vivo*, become infected, even at the peak of virus replication in the lungs (Mengeling et al., 1995; Duan et al., 1997a). Thus, PRRSV appears to have a preference for certain subsets of macrophages *in vivo*. A number of publications described PRRSV antigens in a low number of epithelial cells in bronchi (Pol et al., 1991; Rossow et al., 1995), of epithelial cells in the nasal mucosa (Rossow et al., 1996), of type II pneumocytes (Halbur et al., 1994) and of endothelial cells (Halbur et al., 1995, 1996). In contrast to these findings, Duan et al. (1997a) and Beyer et al. (2000) did not observe PRRSV antigens in these cell types. Since macrophages are ubiquitously distributed cells that display a variety of morphological phenotypes (Rutherford et al., 1993), it is possible that the viral antigen-positive epithelial and endothelial cells represent in fact monocytes/macrophages during their migration through tissues. Indeed, Teifke et al. (2001) and Howerth et al. (2002) recently ruled out the previously postulated role of epithelial and endothelial cells in the replication of PRRSV.

The tropism of PRRSV for pulmonary macrophages has led to the central hypothesis that lung defense mechanisms may be suppressed following PRRSV infection. Indeed, macrophages play important roles both in innate and acquired immunity, performing a large variety of functions that include phagocytosis, killing of micro-organisms, scavenging at sites of tissue injury, processing and presentation of antigens to lymphocytes, and cytokine production. Consequently, several studies have been undertaken to determine whether there is local or systemic immunosuppression following PRRSV infection, but the results of these studies appear to be rather ambiguous. Several researchers have investigated the phagocytic and microbicidal capacity of macrophages following a PRRSV infection, but their results were variable. Chiou et al. (2000) demonstrated that the phagocytic capacity of PAMs against *Candida albicans* was significantly inhibited, whereas the ability of PAMs to internalize *Staphylococcus aureus* or *Escherichia coli* was not affected (Thanawongnuwech et al., 1997; Oleksiewicz & Nielsen, 1999). Galina et al. (1994a), on the other hand, demonstrated that the phagocytic capacity of PAMs against *Streptococcus suis* was increased. The bactericidal activity of macrophages following a PRRSV infection has been shown to be reduced in most studies (Molitor et al., 1992a; Thanawongnuwech et al., 1997; Chiou et al., 2000), but Galina et al. (1994a) reported that PRRSV increases the ability of PAMs for intracellular killing of bacteria. The results of studies, which dealt with the oxidative burst capacity of macrophages following a PRRSV infection, were more uniform. Indeed, all studies revealed that PRRSV decreases the ability of macrophages to release superoxide anions and hydrogen peroxide (Molitor et al., 1992a; Zhou et al., 1992; Thanawongnuwech et al., 1997; Chiou et al., 2000; López-Fuertes et al., 2000a). Nevertheless, Chiou et al. (2000) reported that the amount of production of these oxygen metabolites was enhanced when measured on a per viable cell basis. Thanawongnuwech et al. (1998) demonstrated that PRRSV markedly reduces the clearance capacity of the lungs, since the ability of pulmonary intravascular macrophages to clear copper particles was significantly reduced between 7 and 14 days after inoculation. PRRSV also affects the processing and presentation of antigens to lymphocytes. An enhanced immune response against some pathogens following a PRRSV infection has been described. Molitor et al. (1992b) showed that pseudorabies virus and *Brucella abortus* antibody titres and delayed type hypersensitivity responses were significantly enhanced in PRRSV-infected pigs. The latter finding was confirmed by Albina et al. (1998), who demonstrated that PRRSV did not impair the immune response following pseudorabies

virus vaccination. On the contrary, the total number of white blood cells and the number of IgM^+, $CD2^+$ and $CD8^+$ cells of pigs previously infected with PRRSV and then challenged with virulent pseudorabies virus were increased. Brun et al. (1994) similarly demonstrated that PRRSV enhanced the antibody production after a virulent challenge with swine influenza virus. Another study showed no differences in antibody titres against pseudorabies virus between PRRSV- and non-infected control pigs (De Bruin et al., 2000). Finally, PRRSV also interferes with the production of pro-inflammatory cytokines. López-Fuertes et al. (2000b) demonstrated that PRRSV strongly reduced the interleukin (IL)-1 and tumour necrosis factor-alpha (TNF-α) mRNA expression in PAMs. The latter finding was supported by Chiou et al. (2000), who showed that the level of bioactive TNF-α secretion was markedly reduced soon after PRRSV infection. Zhou et al. (1992), on the other hand, demonstrated that expression of IL-1 was enhanced in PAMs from PRRSV-infected pigs at 1 week after inoculation. In conclusion, research into the macrophage functions following a PRRSV infection is very scanty and inconclusive.

A particular feature of PRRSV is its capacity to persist for long periods of time after initial infection. Evidence for persistence came from several publications. Virus transmission by direct contact between susceptible pigs and 8 to 16 weeks earlier infected pigs have been reported (Zimmerman et al., 1992; Albina et al., 1994; Bilodeau et al., 1994; Bierk et al., 2001; Wills et al., 2002). There are some conflicting data about the exact duration and site of PRRSV replication during the persistent stage. To our opinion, detection of infectious virus, either by virus isolation or swine bioassay, is required to classify animals as persistently infected. Some studies strongly indicate that pulmonary macrophages are the major source of persistent infection. In studies by Duan et al. (1997a) and Beyer et al. (2000), lungs and alveolar macrophages were the only tissues in which PRRSV was persistently detected for 35 days after inoculation. Further evidence for virus persistence in pulmonary macrophages has been obtained by Mengeling et al. (1995) and Shibata et al. (1997). They demonstrated that PAMs obtained by broncho-alveolar lavage were virus-positive up to days 49 or 70 after inoculation. Other studies, however, suggest PRRSV persistence in lymphoid organs, especially in the tonsils. Rossow et al. (1994) could isolate PRRSV exclusively from tonsils, spleen, and lymphoid tissues and not from the lungs at 28 days after inoculation. Further, PRRSV could be isolated from tonsil homogenates until 84 (Allende et al., 2000) to 105 (Horter et al.,

2000) days after inoculation. Wills et al. (1997) reported that infectious virus was isolated up to 157 days after inoculation from oropharyngeal swabs. The latter authors, however, postulated that due to the sampling process, oropharyngeal samples may have consisted of blood traces, saliva, lacrimae, nasal secretions, and respiratory tract secretions. PRRSV is also able to persist in the reproductive tract of boars. Swenson et al. (1994) detected infectious virus in the semen of experimentally infected boars for as long as 43 days following exposure. Using the polymerase chain reaction (PCR), viral RNA in semen has been detected until 92 days after inoculation (Christopher-Hennings et al., 1995a). A PCR was used because virus isolation on semen is troublesome (Christopher-Hennings et al., 1995b). The source of PRRSV in semen is unknown, but PRRSV has been detected in the bulbo-urethral gland until 101 days after inoculation (Christopher-Hennings et al., 1995a) or in testes until 25 days after inoculation (Sur et al., 1997).

1.1.6. Disease

PRRS initially emerged as a devastating disease, characterized by reproductive failure in pregnant sows and gilts and respiratory disease problems in pigs of all ages, but particularly in nursery pigs. Later on, it became clear that the major characteristic of the disease is a high variability of clinical signs with a wide range of severity of clinical signs, including a subclinical course of infection.

During its epizootic phase, PRRS in sow herds was characterized by reproductive disorders, including late-term abortions, early farrowings and the birth of weak and stillborn pigs (Ohlinger et al., 1991; Wensvoort et al., 1991). Sometimes these signs were clearly evident but often they were only detectable after analyzing detailed herd records. Decreased farrowing rates and a delayed return to oestrus were persistent features. A less common finding described in Europe, but not in the United States was a transient discoloration (cyanosis) of the ears, abdomen and vulva (Wensvoort, 1993). On a farm basis, the reproductive disorders lasted 1 to 4 months. Concurrently, the disease in young pigs was characterized by respiratory disorders, increased pre-weaning mortality and growth retardation (Ohlinger et al., 1991; Wensvoort et al., 1991). These clinical signs were largely age-dependent, i.e. they were usually more severe in nursery pigs and mild in fattening pigs.

Currently, PRRSV is enzootic in most pig-producing countries throughout the world and it is generally accepted that most PRRSV infections in breeding pigs are subclinical. However, in some infected herds, periodic reproductive failure in breeding pigs and recurrent respiratory disease problems in nursery and fattening pigs have been reported (Keffaber et al., 1992; Stevenson et al., 1993; Zeman et al., 1993; Dee & Joo, 1994; Done & Paton, 1995). Furthermore, veterinarians and farmers have reported an increase in respiratory disease problems and poor productivity in nursery and fattening pigs since the enzootic appearance of PRRSV (Done & Paton, 1995). In the majority of experimental PRRSV infection studies, overt respiratory signs and poor doing were difficult to reproduce. The most prominent finding was a transient fever 2 to 3 days after inoculation (Pol et al., 1991; Paton et al., 1992; Plana-Durán et al., 1992; Ramos et al., 1992; Yoon et al., 1992; Albina et al., 1994; Van Reeth et al., 1996; Segalés et al., 1999). In some studies, anorexia and depression have also been recorded (Fichtner et al., 1993; Depner et al., 1999). More severe respiratory signs have been observed with some North American isolates (Halbur et al., 1993; Rossow et al., 1994; Halbur et al., 1995; Loemba et al., 1996). Experimental inoculations with other North American isolates remained, however, subclinical (Mengeling et al., 1996). Thus, some North American isolates are seemingly more virulent than others and than the European Lelystad virus (Halbur et al., 1995). In conclusion, it can be stated that respiratory disease problems under field circumstances, especially in Europe, can hardly be attributed to single PRRSV infections and that other factors have to be involved.

In herds with respiratory disease problems, PRRSV has been isolated in combination with a mixture of bacteria, including *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Haemophilus parasuis*, *Arcanobacterium pyogenes*, *Pasteurella multocida, Escherichia coli, Salmonella* Choleraesuis, and *Streptococcus suis*, and in combination with common respiratory viruses, such as swine influenza viruses and porcine respiratory coronavirus (Hopper et al., 1992; Morrison et al., 1992; Halbur et al., 1993; Stevenson et al., 1993; Zeman et al., 1993; Kay et al., 1994; Done & Paton, 1995; Kamogawa et al., 1996; Kawashima et al., 1996; Kobayashi et al., 1996). These observations supported the idea that PRRSV may cause respiratory disease problems and/or poor doing in combination with other infectious agents. This has stimulated research into the combined effects of PRRSV and other pathogens. Consequently, a large number of workers have investigated the effects of dual infections

with PRRSV and bacteria and/or viruses, but their findings were rather ambiguous. A summary of their findings is listed in Table 1.

Table 1. Clinical, pathological and microbiological effects of experimental dual infections with PRRSV and bacteria or viruses.

Secondary agent	Authors	Combination	Effect on …		
			disease*	lesions*	bacterial colonization*
Haemophilus parasuis	Cooper et al., 1995	PRRSV-7 d-*H. parasuis*	-	-	-
	Solano et al., 1997	PRRSV-5 d-*H. parasuis*	-	-	-
	Segalés et al., 1999	PRRSV-5 d-*H. parasuis*	-	not studied	-
	Brockmeier et al., 2002	PRRSV-7 d-*H. parasuis*	-	-	+
Pasteurella multocida	Cooper et al., 1995	PRRSV-2 or 7 d-*P. multocida*	-	-	-
	Carvalho et al., 1997	PRRSV-5 d-*P. multocida*	-	-	not studied
	Brockmeier et al., 2001	PRRSV-7 d-*P. multocida*	-	-	-
Streptococcus suis	Galina et al., 1994b	PRRSV-7 d-*S. suis*	+	+	+
	Cooper et al., 1995	PRRSV-2 or 7 d-*S. suis*	-	-	-
	Thanawongnuwech et al., 2000	PRRSV-7 d-*S. suis*	+	+	+
	Halbur et al., 2000	PRRSV-7 d-*S. suis*	+	+	+
	Schmitt et al., 2001	PRRSV-7 d-*S. suis*	+	+	+
Salmonella Choleraesuis	Cooper et al., 1995	PRRSV-7 d-*S.* Choleraesuis	-	-	-
	Wills et al., 2000	*S.* Choleraesuis-3 d-PRRSV	+	-	+
Actinobacillus pleuropneumoniae	Pol et al., 1997a	PRRSV-7 d-*A. pleuropneumoniae*	not studied	+	not studied
Bordetella bronchiseptica	Brockmeier et al., 2000	PRRSV-0 d-*B. bronchiseptica*	+	+	+
Mycoplasma hyopneumoniae	Albina et al., 1995	PRRSV-21 d-*M. hyopneumoniae*	-	-	not studied
		M. hyopneumoniae-21 d-PRRSV	-	-	not studied
	Van Alstine et al., 1996	PRRSV-7 d-*M. hyopneumoniae*	-	-	-
	Thacker et al., 1999	PRRSV-0 or 10 d-*M. hyopneumoniae*	+	+	-
		M. hyopneumoniae-21 d-PRRSV	+	+	-

Table 1. *Continued*.

Secondary agent	Authors	Combination	Effect on …		
			disease*	lesions*	bacterial colonization*
influenza virus	Brun et al., 1994	PRRSV-0 or 4 d-H1N1	-	not studied	Not applicable
	Pol et al., 1997a	PRRSV-7 d-H3N2	not studied	-	
	Van Reeth et al., 1996	PRRSV-3 d-H1N1	+	not studied	
	Lee et al., 1999	PRRSV-7 d-H1N1	not studied	+	
	Van Reeth et al., 2001	PRRSV-3 d-H1N1	+	not studied	
		PRRSV-7 d-H1N1	+	not studied	
		PRRSV-14 d-H1N1	-	not studied	
porcine respiratory coronavirus	Van Reeth et al., 1996	PRRSV-3 d-PRCV	+	not studied	
transmissible gastroenteritis virus	Wesley et al., 1998	PRRSV-14 d-TGEV	-	not studied	
porcine circovirus type 2	Harms et al., 2000	PRRSV-0 d-PCV type 2	+	+	
	Allan et al., 2000	PRRSV-0 d-PCV type 2	-	+	
	Rovira et al., 2002	PRRSV-7 d-PCV type 2	+	+	
pseudorabies virus	Chen et al., 1998	PRRSV-10 d-PRV	+	+	
classical swine fever virus	Depner et al., 1999	PRRSV-3 d-CSFV	-	not studied	

*+: aggravating effect; -: no effect

Dual infections of PRRSV with bacteria

Haemophilus parasuis - Cooper et al. (1995) were unable to potentiate infections by super-exposure of four- to five-week-old specific pathogen-free (SPF) pigs to *Haemophilus parasuis* after inoculation with a North American PRRSV strain. Solano et al. (1997) and Segalés et al. (1999) showed also that a dual infection of conventional pigs with PRRSV followed by *Haemophilus parasuis* did not result in an increased bacterial polyserositis and replication as compared to their controls. Brockmeier et al. (2002), on the other hand, demonstrated that PRRSV increased the colonization of the upper respiratory tract with *Haemophilus parasuis*. Furthermore, the PRRSV infection predisposed the pigs to a pulmonary infection with *Haemophilus parasuis*.

Pasteurella multocida - A dual infection with PRRSV and *Pasteurella multocida* did not enhance lung lesions and had little effect on bacterial colonization of the lungs (Cooper et al., 1995; Carvalho et al., 1997; Brockmeier et al., 2001).

Streptococcus suis - Galina et al. (1994b), using a virulent strain of *Streptococcus suis* in SPF pigs, observed that only pigs, which had previously been inoculated with PRRSV, developed severe clinical signs, a suppurative meningitis and large numbers of bacteria in several tissues, including the brains and meninges. The latter findings were supported by Thanawongnuwech et al. (2000), Halbur et al. (2000) and Schmitt et al. (2001). Two- to four-week-old conventional pigs, which were inoculated with PRRSV followed 7 days later by *Streptococcus suis*, exhibited more frequent and severe clinical central nervous system disease and lesions typical of a *Streptococcus suis* infection. They had also more widespread tissue dissemination of the bacteria, including the lungs, had a severe decrease in pulmonary copper clearance, and experienced significantly higher mortality than pigs infected with *Streptococcus suis* alone. Feng et al. (2001) reported that piglets infected *in utero* with PRRSV at 98 days of gestation and challenged with *Streptococcus suis* 5 days after birth displayed significantly higher mortality rates and infections of joints and brains compared to controls. In contrast, Cooper et al. (1995) were unable to potentiate infections by super-exposure with *Streptococcus suis* in PRRSV-infected pigs.

Salmonella Choleraesuis – Cooper et al. (1995) reported that a dual infection with PRRSV and *Salmonella* Choleraesuis did not enhance lung lesions and had no effect on bacterial colonization of the lungs. In contrast, Wills et al. (2000) reported that pigs which were dually infected with PRRSV and *Salmonella* Choleraesuis exhibited unthriftiness, rough hair coats, dyspnoea and diarrhea, whereas no clinical signs were observed in pigs inoculated with only PRRSV or only *Salmonella* Choleraesuis.

Actinobacillus pleuropneumoniae - Pol et al. (1997a) obtained ambiguous results in dual infections of PRRSV and *Actinobacillus pleuropneumoniae* in SPF pigs. *Actinobacillus pleuropneumoniae* lesions were enhanced in dually infected pigs compared to single *Actinobacillus pleuropneumoniae*-infected pigs in one experiment, but the authors were unable to repeat their results upon two subsequent attempts.

Bordetella bronchiseptica - Brockmeier et al. (2000) showed that the clinical outcome, including respiratory signs, fever and decreased weight gain, was far more pronounced in pigs inoculated with PRRSV and *Bordetella bronchiseptica* than in pigs inoculated with either organism alone. Furthermore, the PRRSV infection predisposed the pigs to a pulmonary infection with *Bordetella bronchiseptica*. Brockmeier et al. (2001) further demonstrated that a co-infection with PRRSV and *Bordetella bronchiseptica* predisposed pigs to infection of the upper respiratory tract and lungs with *Pasteurella multocida*.

Mycoplasma hyopneumoniae - Attempts to demonstrate an interaction between PRRSV and *Mycoplasma hyopneumoniae* have shown contradictory results. Albina et al. (1995) showed no increased prevalence of clinical signs in PRRSV-*Mycoplasma hyopneumoniae* dually infected pigs. The latter finding was confirmed by Van Alstine et al. (1996), who showed that inoculation of pigs with *Mycoplasma hyopneumoniae* seven days after inoculation with a North American PRRSV strain did not result in exacerbation of the disease. Thacker et al. (1999), on the other hand, showed that pigs, which had been inoculated with both PRRSV and *Mycoplasma hyopneumoniae,* had more severe respiratory signs than pigs inoculated with PRRSV or *Mycoplasma hyopneumoniae* alone. At 4 to 5 weeks after PRRSV inoculation, *Mycoplasma hyopneumoniae*-infected pigs still exhibited lesions typical of PRRSV-induced pneumonia, whereas the lungs of pigs, which had received only PRRSV, were essentially normal.

Dual infections of PRRSV with viruses

Orthomyxoviridae - Van Reeth et al. (1996,2001) demonstrated that Lelystad virus interacted with H1N1-influenza virus to produce more severe disease. Combined PRRSV-H1N1 infections in ten-week-old conventional pigs resulted in enhanced fever, respiratory distress and growth retardation as compared to the respective single virus infections. However, the clinical effects of the dual PRRSV-H1N1 infections were not reproducible to the same level in subsequent groups of experimental pigs. Lee et al. (1999) reported that dual infection of conventional pigs with PRRSV followed by H1N1-influenza virus seven days later resulted in more severe lung lesions. Brun et al. (1994),

on the other hand, failed to induce more severe clinical signs in PRRSV-H1N1 dually infected pigs. Pol et al. (1997a) likewise demonstrated that dual infection of SPF pigs with PRRSV followed by H3N2-influenza virus seven days later did not result in more severe lung lesions.

Coronaviridae – Van Reeth et al. (1996) demonstrated that dual infection of ten-week-old conventional pigs with PRRSV and porcine respiratory coronavirus resulted in enhanced fever, respiratory signs and growth reduction compared to the respective single virus controls. Wesley et al. (1998) demonstrated that the clinical course of transmissible gastroenteritis virus (TGEV) disease was not markedly affected by infection of pigs with TGEV two weeks after they had been infected with PRRSV.

Circoviridae – Several reports have demonstrated that dual infections of pigs with PRRSV and porcine circovirus (PCV) type 2 significantly increased the severity of PRRSV-induced lung lesions (Harms et al., 2000; Allan et al., 2000; Rovira et al., 2002). Pesch et al. (2000) reported that co-infections with PRRSV and PCV type 2 caused severe respiratory signs, which were similar to those observed in pigs with proliferative necrotizing pneumonia.

Herpesviridae - Chen et al. (1998) demonstrated that dual infection of SPF pigs with PRRSV followed by pseudorabies virus (PRV) resulted in an enhanced severity of a PRV infection. Pigs inoculated with PRV only showed depression, whereas dually inoculated pigs showed ataxia, convulsion, peddling and death.

Pestiviridae - Depner et al. (1999) reported that inoculation of conventional pigs with classical swine fever virus during the early phase of a PRRSV infection did not significantly potentiate the clinical course of classical swine fever.

Thus, the results of these studies ranged from no interaction to increased incidence and severity of disease, sometimes with conflicting results for the same combination of infectious agents. This ambiguity may be due to a number of factors, including differences in the age of the pigs, the virus dose, the inoculation method, the strain of PRRSV and/or the secondary pathogen, the timing of infections, and the sanitary status of the pigs. Van Reeth et al. (2001) demonstrated that the interval between a PRRSV and a subsequent H1N1-influenza virus infection can determine the clinical outcome. Though PRRSV replicates in the lungs for long periods of time (Mengeling et al., 1995; Duan et al., 1997a), disease was only observed if H1N1-influenza virus inoculation occurred sooner than 14 days after PRRSV inoculation. The latter authors also reported that only

20% of caesarean-derived colostrum-deprived pigs developed typical clinical signs after an experimental PRRSV-H1N1 influenza virus dual infection, whereas in conventional pigs, morbidity was 100% and disease severity was higher. Thus, it appears that severe clinical signs develop more readily in pigs of a lower sanitary status.

1.1.7. Immunity

PRRSV-infected pigs develop a humoral immune response that can easily be detected by the presence of serum antibodies to the virus. Most serologic assays indicate that serum antibodies appear at 1 to 2 weeks after infection, reach a maximum by 5 to 6 weeks and persist for 42 weeks. Both immunoglobulins M and G are involved in the specific humoral immune response to PRRSV. IgM antibodies are first detected at day 7, peak at 14 to 21 days and rapidly decrease, being undetectable by 35 to 42 days. IgG antibodies appear by day 11 to 14 after infection, peak at 21 to 28 days and are detectable for several months (Loemba et al., 1996; Vézina et al., 1996). Antibodies with a virus-neutralizing activity appear more slowly. They are usually detected first at 4 to 6 weeks after infection and reach a maximal titre about 10 to 12 weeks after infection (Morrison et al., 1992; Yoon et al., 1995; Loemba et al., 1996; Albina et al., 1998; Wu et al., 2001). Meier et al. (2000) detected virus-neutralizing antibodies not earlier than 11 to 13 weeks after infection. Studies of the immune response to individual viral proteins have shown that the antibody responses are mounted primarily to the nucleocapsid protein (N) and, to a lesser extent, to the matrix (M) and the major envelope (E or GP_5) proteins (Meulenberg et al., 1995; Loemba et al., 1996; Kwang et al., 1999). Antibodies to GP_5 were demonstrated as early as 6 days after infection, whereas antibodies to the N and M proteins were detected only by the end of the second week after infection (Loemba et al., 1996; Kwang et al., 1999). Antibodies to the 2b protein appeared by 18 days after infection, and by 39 days all pigs had antibodies against this protein (Wu et al., 2001). The differences in antibody responses to these viral proteins have been attributed to their relative portion within the virion, rather than to their immunogenicity (Loemba et al., 1996).

The role of humoral immunity in protection upon challenge is questionable, since a common feature of a PRRSV infection is the co-existence of viraemia and antibodies (Rossow et al., 1994; Halbur et al., 1996; Duan et al., 1997a). Furthermore, it has been demonstrated that with a few North American PRRSV isolates early antibodies rather

enhance than inhibit virus infection through Fc receptor-mediated attachment of virus-antibodies complexes to susceptible macrophages. This process is recognized as "antibody-dependent enhancement of infectivity (ADEI)" (Choi et al., 1992; Christianson et al., 1993; Yoon et al., 1994; Yoon et al., 1996). The possibility to isolate virus in spite of the presence of neutralizing antibodies indicates that neutralizing antibodies are not the only immune components involved in the complete elimination of the virus. The immune system of the host is ultimately able to clear PRRSV and seems to do it in the majority of the animals by 150 days after inoculation or shortly thereafter (Allende et al., 2000). The mechanisms by which the virus is finally cleared are not known.

Little is known about the cellular immune response to a PRRSV infection. Bautista & Molitor (1997) and López-Fuertes et al. (1999) demonstrated a clear T lymphocyte proliferation to PRRSV. This T lymphocyte proliferation was first detected at 4 weeks after infection, peaked at 7 weeks and appeared to decline after 11 weeks. The proliferation involved mainly $CD4^+$ T lymphocytes, but also $CD8^+$ T lymphocytes. An elevated level of $CD8^+$ T lymphocytes from 3 to 5 weeks after infection has been demonstrated by several researchers (Shimizu et al., 1996; Albina et al., 1998; Janutenaite et al., 2002). The biological significance of this change in $CD8^+$ cell numbers is not clear, but in the study of Albina et al. (1998), viraemia started to decline shortly after the proliferation of these $CD8^+$ cells. Bautista et al. (1999) demonstrated that the T lymphocyte proliferation response was largest after stimulation with the product of open reading frame (ORF) 6, suggesting that the matrix protein (M) is specifically recognized by T lymphocytes from PRRSV-infected pigs. In another study, performed by Meier et al. (2000), T lymphocyte proliferation was first detected at 3 weeks after infection and appeared to increase steadily from 11 to 13 weeks. The latter authors also reported that the frequency and quality of a T lymphocyte-mediated interferon-gamma (IFN-γ) response, as detected by ELISPOT, was low and very poor during the first 9 to 10 weeks after infection.

1.1.8. Vaccination

The important economic impact of PRRSV on the swine industry (Polson et al., 1992) has led to the development of PRRSV vaccines. Because of safety reasons, the very first vaccines consisted of inactivated viruses. These vaccines, however, appeared to lack immunogenicity, resulting in a poor protection against a challenge with wild-type virus (Plana-Durán et al., 1997a). Subunit vaccines were also constructed, consisting of ORFs

incorporated into baculoviruses to produce the protein *in vitro* (Plana-Durán et al., 1997b; Kwang et al., 1999; Qiu et al., 2000). The baculovirus ORF7 product induced antibodies upon vaccination, but did not provide immunity against a challenge with wild-type PRRSV (Plana-Durán et al., 1997b). Pigs, immunized with a plasmid encoding GP_5, on the other hand, were protected from viraemia upon challenge with wild-type PRRSV (Pirzadeh & Dea, 1998). Rogan et al. (2000) were able to elicit neutralizing antibodies in pigs immunized with a vacciniavirus ORF2 product.

Today, several modified live vaccines, developed by different companies, have been licensed and are commercially available for use in feeder pigs and sows. Vaccines containing either European or North American serotypes are available. All these vaccines have been evaluated for efficacy and safety.

Efficacy testing of modified live vaccines

With regard to the efficacy of modified live PRRSV vaccines, there is one major issue of concern: the degree of cross-protection between the European and the North American serotype.

When a vaccine is claimed to protect against PRRSV-induced respiratory problems, a vaccination/challenge model in young pigs is performed. Owing to the fact that clinical signs are mostly absent among non-vaccinated challenge control pigs (van Woensel et al., 1998; Labarque et al., 2000), it is not possible to assess the efficacy of vaccines based on clinical signs alone. Therefore, the efficacy of vaccination is usually assessed by determining the reduction in viraemia after challenge with a virulent virus. These studies have clearly demonstrated that the virological protection was significantly better after a homologous challenge than after a heterologous one. Vaccination with a North American strain suppressed the replication of European wild-type virus to some extent, but a far more effective suppression of replication of the challenge virus was obtained in pigs vaccinated with a European strain (van Woensel et al., 1998; Labarque et al., 2000). When the challenge was performed with a North American wild-type virus, the mean cumulative virus titres in blood were clearly reduced in pigs vaccinated with a North American serotype vaccine when compared with those of non-vaccinated challenge control pigs and pigs vaccinated with a European serotype vaccine (Labarque et al., 2000). On the other hand, Nielsen et al. (1997) demonstrated an effective suppression of replication of the challenge virus in pigs, vaccinated with a North American serotype vaccine and challenged with a European wild-type virus. In this study, the vaccine was

administered intranasally instead of parenterally. Nodelijk et al. (2001) reported that, despite the fact that both the duration and the level of viraemia upon a heterologous challenge were significantly reduced in pigs vaccinated with a North American serotype vaccine, no reduction of transmission of virulent PRRSV was obtained by the vaccination.

When a vaccine is claimed to protect against PRRSV-induced reproductive failure, a vaccination/challenge model in pregnant sows is performed. Since sow studies are expensive and difficult to manage, most of them have been performed with a low number of sows. Sows, immunized with a North American serotype PRRSV vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica) 3 to 9 weeks before breeding and challenged at approximately 90 days of gestation with virulent PRRSV strains, showed a clearly better farrowing performance compared to non-immunized challenge control sows (Gorcyca et al., 1996; Benson et al., 2000; Medveczky et al., 2002). Benson et al. (2000) and Medveczky et al. (2002) reported that still a considerable number of the piglets of the immunized sows were viraemic at birth. Though, the immunized sows gave birth to less viraemic piglets than the non-immunized challenge control sows (8 to 10% versus 42 to 44%). Hesse et al. (1996a) did not observe viraemic piglets when sows were immunized with another North American serotype PRRSV vaccine (Prime Pac® PRRS, Schering Plough Animal Health Corporation) 4 to 6 weeks before breeding and challenged at 85 days of gestation with a North American wild-type strain. When the challenge was performed with a European wild-type strain, this resulted in the birth of stillborn pigs, mummies and viraemic pigs (Hesse et al., 1996b). The immunized sows gave birth to 27% dead piglets and 5% viraemic piglets, whereas the percentages in non-immunized challenge control sows were 64 and 69%, respectively. Scortti et al. (1999) reported that when sows, immunized 3 weeks before breeding with either of two European serotype PRRSV vaccines (Amervac-PRRS®, Hipra Laboratorios and Pyrsvac-183®, Syva Laboratorios, respectively), were challenged with European wild-type strains at 90 days of gestation, 7 and 24% of the offspring, respectively, were viraemic. Mengeling et al. (1999) reported that 4% of the offspring of sows, immunized with Ingelvac® PRRS MLV at 60 days of gestation and challenged with a North American wild-type strain 30 days later, were viraemic. When the challenge was performed with a European wild-type strain, 32% of the piglets were viraemic (Canals et al., 2000). Non-immunized challenge control sows in these studies gave birth to 57 (Mengeling et al., 1999) and 76% (Canals et

al., 2000) viraemic piglets. In conclusion, results of these studies indicate that vaccination cannot completely avoid the transplacental transmission of challenge virus to piglets. Nevertheless, the farrowing performance in vaccinated sows and gilts was clearly better than that of non-vaccinated ones.

Safety testing of modified live vaccines

The main safety concerns relating to modified live PRRSV vaccines are (i) pathogenicity of the vaccine strain, (ii) dissemination of the vaccine throughout the body and ability to spread, (iii) reversion to virulence and (iv) recombination with field strains.

(i) Pathogenicity of the vaccine strain

It has been reported that clinical signs and lesions caused by vaccine viruses are minimal. It has been shown by Kang et al. (1996) that Ingelvac® PRRS MLV does not induce clinical signs after parenteral administration. Opriessnig et al. (2002) further demonstrated that this vaccine virus does not induce lung lesions after intranasal administration. Similarly, it has been demonstrated that Porcilis® PRRS (Intervet NV) does not induce disease after parenteral administration (Stadejek & Pejsak, 1998; Astrup & Riising, 2002).

(ii) Dissemination of the vaccine throughout the body and ability to spread

No much information is available about the dissemination of vaccine viruses throughout the body after vaccination. It has been demonstrated that vaccine viruses can be isolated from blood (Christopher-Hennings et al., 1996; Bøtner et al., 1997; Stadejek & Pejsak, 1998; Astrup & Riising, 2002), but information about the replication of vaccine viruses in target organs, such as lymphoid organs and lungs, is scarce. Thacker et al. (2000) reported that Ingelvac® PRRS MLV can be isolated from lungs after parenteral administration. After vaccinating boars, vaccine virus can be detected in semen (Christopher-Hennings et al., 1996; Bøtner et al., 1997; Nielsen et al., 1997).

Ingelvac® PRRS MLV has been shown to be shed and infect naive contact pigs held in the same pen and in adjacent pens (Torrison et al., 1996). The latter authors also reported transmission to a non-adjacent pen, raising the possibility of aerosol transmission. Bøtner et al. (1997) reported spread of this vaccine virus to non-vaccinated sows and subsequent transplacental infection of fetuses. Stadejek & Pejsak (1998) reported no spread of

Porcilis® PRRS from vaccinated pigs to sentinel pigs, since no seroconversion was observed in any of the sentinels. Sipos et al. (2002) and Astrup & Riising (2002), on the other hand, demonstrated that this vaccine virus has ability to spread from vaccinated to susceptible pigs.

(iii) Reversion to virulence

PRRSV is an RNA virus and as compared to DNA viruses, RNA viruses have a tendency to mutate at a high rate. When these mutations occur in vaccine strains, this can possibly result in an increased virulence. Recently, it was indeed demonstrated that Ingelvac® PRRS MLV can undergo mutations under field conditions (Storgaard et al., 1999; Nielsen et al., 2001). The implications of these findings, however, are still under debate.

(iv) Recombination with field strains

Recombination with viruses is possible when the host cell is simultaneously infected with two different strains of the same virus. Thus, simultaneous infection of a pig with a vaccine and a field strain of PRRSV could theoretically lead to recombination of genetic material. Bøtner et al. (1999) postulated that recombination is very unlikely between European and North American type PRRSV isolates, because of their large genetic and antigenic differences. Murtaugh et al. (2002) demonstrated that genetic recombination between two attenuated PRRSV vaccine strains can occur *in vitro* in PAMs and in MA-104 cells, but they could not confirm their findings *in vivo*. The theoretical recombination of a vaccine and a field strain could only result in a strain that is not more virulent than the original field strain. Recombination of vaccine strains with a field strain has not been reported until now.

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1.2. Lipopolysaccharides and role in respiratory disease

1.2.1. Introduction

Respiratory disease in pigs has a multi-factorial background and several infectious agents, both viral and bacterial, have been associated with this problem. However, non-infectious airborne contaminants may also contribute to the development of respiratory disease in swine. The major airborne contaminants in swine confinement buildings are organic dust, toxic gases (ammonia and hydrogen sulphide being the most important), and biologically active components such as peptidoglycans, beta-1,3-glucans and endotoxins (Donham, 1994). Epidemiological studies have documented a close relationship between endotoxin concentrations and the prevalence of respiratory disease in both pigs and pig farmers (Donham, 1991; Heederik et al., 1991). Exposure to endotoxins can occur in a number of ways. Massive amounts of endotoxins are released locally in the lungs during infections with Gram-negative bacteria (Pugin et al., 1992). The treatment of Gram-negative bacterial infections with antibiotics may also result in the release of endotoxins in the lungs (Morrison, 1998). Finally, endotoxins are present in dust in swine units at concentrations ranging from 0.04 to 1.2 $\mu g/m^3$ air (Rylander, 1994; Zejda et al., 1994). These concentrations may increase during activities, such as cleaning, feeding, moving or sorting of animals (Donham, 1990; Malmberg & Larsson, 1993; Donham, 1994). Since airborne endotoxins are bound to cell membranes, endotoxin concentrations are often underestimated by the standard Limulus Amoebocyte Lysate (LAL) assay (Rylander et al., 1989). When measured with gas chromatography-mass spectrometry, endotoxin concentrations up to 4.9 $\mu g/m^3$ air were measured in dust in swine units during weighing activities (Zhiping et al., 1996).

1.2.2. Structure of lipopolysaccharides

Endotoxins are a major constituent of the cell wall of Gram-negative bacteria (Rylander, 1994). Lipopolysaccharide (LPS) is the main component of endotoxin and it is formed by a glucosamine-based phospholipid, called lipid A, that is covalently linked to a hydrophilic heteropolysaccharide (Rietschel et al., 1994). Lipid A is responsible for most of the toxic properties of endotoxin (Rietschel et al., 1993). The polysaccharide part

is made up of two parts, an oligosaccharide with a composition varying with bacterial species (O-specific chain) and a rather invariable core section, which is located between the oligosaccharide and the lipid A. The chemical structure of LPS is shown in Figure 1.

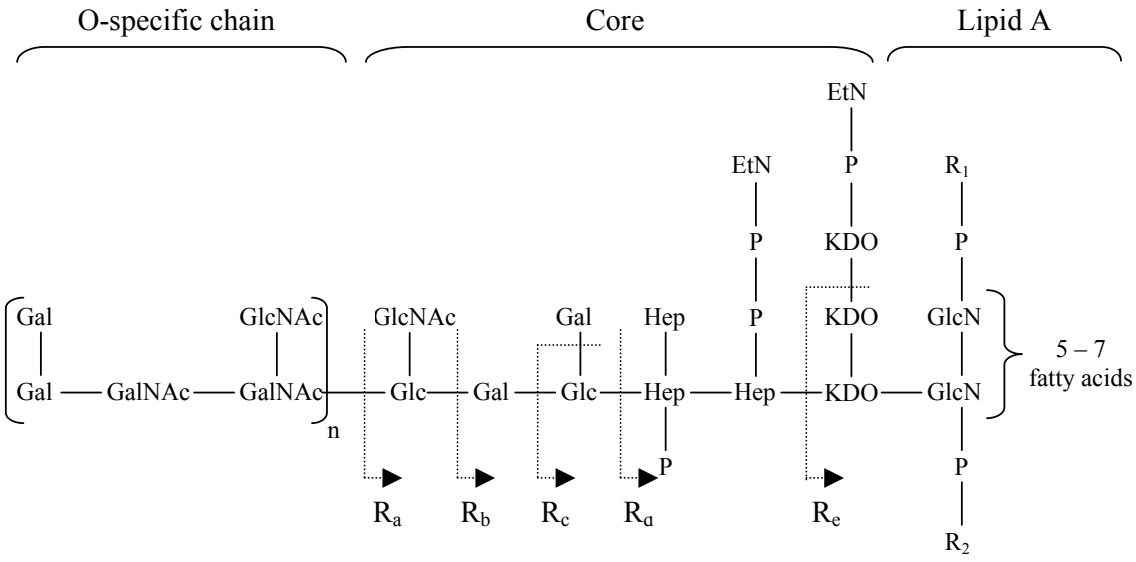

Fig. 1. Structure of *Salmonella* LPS.

Gal: galactose; GalNAc: N-acetyl-galactosamine; Glc: glucose; GlcNAc: N-acetyl-glucosamine; Hep: heptose; P: phosphate; EtN: ethanolamine; KDO: 2-keto-3-deoxyoctonoic acid; R_1 and R_2: phosphoethanolamine or aminoarabinose; R_a to R_e indicate incomplete forms of LPS.

Although the terms endotoxin and LPS are often used interchangeably, the two terms are not synonymous. Endotoxin refers to the Gram-negative bacterial cell wall containing the LPS and other naturally occurring compounds in the bacterial cell wall. Thus, exposure under real life conditions also includes bacterial proteins and other bacterial cell wall constituents. LPS, on the other hand, implies a chemically purified endotoxin with no or only trace amounts of bacterial cell wall proteins, obtained by extraction procedures, and not present in organic dust (Rylander, 1994). LPS is used in most experimental exposure studies.

1.2.3. Cellular signaling pathway of lipopolysaccharides

LPS exert their biological effects through interaction with specific receptors. Monocytes/macrophages and epithelial cells have been identified as the primary target cells for LPS (Thorn, 2001). LPS first binds to the LPS-binding protein (LBP) and is then delivered to the cell surface receptor CD14. Next, LPS is transferred to the transmembrane signaling Toll-like receptor 4 (TLR4) and its accessory protein MD2. LPS then stimulates the activation of various intracellular signaling pathways. These signaling pathways directly or indirectly phosphorylate and activate various transcription factors, which coordinate the induction of many genes encoding inflammatory mediators such as cytokines, chemokines, arachidonic acid metabolites, and oxygen metabolites (*reviewed by* Guha & Mackman, 2001).

1.2.4. Biological effects of lipopolysaccharides

Systemic and respiratory responses to LPS have been extensively investigated after intravenous administration, but we will focus on the effects of LPS when inhaled or administered to the lungs by either nebulization or direct intratracheal administration. The effects of LPS on the respiratory health of pigs, which often are continuously exposed throughout their lifetime, have not been studied extensively. Indeed, most LPS inhalation-challenge studies have been performed in small laboratory animals and humans. Nevertheless, we will focus on the biological effects of LPS in humans and pigs.

Clinical signs

Humans – In humans, LPS has been shown to cause influenza-like symptoms (Rylander et al., 1989; Michel et al., 1997). Chronic coughing, excessive sputum and phlegm production, chest tightness, wheezing, and dyspnoea are common respiratory symptoms. In addition, general symptoms, such as fever, headache, tiredness, chilling, joint and muscle pain, nausea, eye irritation, and malaise have been reported. The doses needed to induce such symptoms under experimental conditions were high, especially when compared with endotoxin levels found in dust in field studies (Rylander, 1997). This discrepancy may be due to the bioavailability of endotoxin and/or to the presence of other agents in dust (Thorn, 2001). Most often, clinical recovery occurs spontaneously within a

few days after withdrawal from exposure. Nevertheless, continuous exposure to endotoxins is thought to be responsible for a number of occupational diseases including occupational asthma (Donham et al., 1989), byssinosis (Rylander, 1982), swine workers's disease (Donham et al., 1989) and farmers's lung disease (Thelin et al., 1984).

Pigs – The clinical effect of inhalation or intratracheal inoculation of LPS in pigs has not been documented extensively. Except for infrequent coughing, no clinical signs have been observed after nebulization of LPS at concentrations found in pig buildings (Urbain et al., 1999). After intrabronchial LPS administration (dosage 200 μg/kg body weight), pigs appeared very weak, but no increase in body temperature was recorded (Urbain et al., 1996). When very high doses of LPS (1 mg/kg body weight) were administered to specific-pathogen free (SPF) pigs by intratracheal route, general signs, including fever, depression, anorexia, and transient vomiting and diarrhea were reported (Liggett et al., 1986). The latter authors did not report respiratory signs.

Lung functional changes

Humans – Studies in humans have demonstrated that inhalation of LPS leads to impaired lung function. A decrease in forced expiratory volume in one second and forced vital capacity has been reported (Vogelzang et al., 1998). Although the decrease of both lung function parameters is suggestive of an obstructive type of lung disease, other researchers observed lung functional changes indicative of a restrictive pattern (Herbert et al., 1992; Michel et al., 1995). The latter authors demonstrated a reduction of the alveolar-capillary diffusion capacity after inhalation of LPS. In total, the balance of studies in humans suggests development of obstructive rather than restrictive pulmonary disease.

Pigs – To our knowledge, no information is available about lung functional changes following LPS exposure in pigs.

Lung inflammatory changes

Humans – Exposure of humans to LPS can induce inflammatory responses in the lungs without any effect on the lung functions. If high amounts of LPS (50 μg/kg body weight) are administered to humans, clinical signs and decreased lung function are observed, whereas smaller LPS doses (5 μg/kg body weight) cause a mild inflammation only (Michel et al., 1997). The most prominent inflammatory change following LPS exposure is a rapid and massive influx of inflammatory cells, mainly neutrophils, into the airways

(Snella & Rylander, 1982; Sandström et al., 1992; Wesselius et al., 1997). Significant increases have also been found for macrophages and lymphocytes (Sandström et al., 1992; Michel et al., 1997). The cellular influx is associated with an increase in broncho-alveolar lavage (BAL) fibronectin, interleukin (IL)-8 and leukotriene B4, which are all chemoattractants for neutrophils and other cells (Sandström et al., 1992; Wang et al., 1997; Nichtingale et al., 1998). LPS exposure also results in the production of different pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and IL-1 (Clapp et al., 1994; Jagielo et al., 1996; Wesselius et al., 1997). Many of the biological effects of LPS may be attributed to the release of these cytokines. These cytokines indeed mediate leukocyte recruitment and activation in the lungs, increased lung microvascular permeability and pulmonary dysfunctions (Bielefeldt-Ohmann, 1995).

Pigs – There are few publications on the lung inflammatory response to inhalation or direct intratracheal administration of LPS in pigs. Holst et al. (1994) reported increased numbers of neutrophils in BAL fluid of pigs, exposed to dustborne endotoxins (8.6 μg/m^3 air) and fed endotoxin-contaminated feed (100 mg/pig), but the effect of inhalation was not evaluated separately. Jolie et al. (1998) reported that 15 weeks of exposure (5 days/week; 8 hours/day) to feed dust with artificially added endotoxin (42 ng/m^3) led to an increased neutrophil count in BAL fluid. Liggett et al. (1986) reported airway infiltration by both neutrophils and macrophages 24 hours after intratracheal administration of LPS to six-week-old SPF pigs, but only when very high doses were used (1 mg/kg body weight). Urbain et al. (1996) similarly reported an increase in the number of neutrophils and macrophages in BAL fluid after nebulization of LPS (400 μg/kg body weight). According to the latter authors, the cellular response was 30 times higher after intrabronchial administration than after nebulization. That a more moderate reaction was obtained after nebulization of LPS was ascribed both to the ability of the upper airways to act as a filter and/or to the fact that the LPS is dispersed and thus diluted throughout the respiratory tract after aerosolization. Urbain et al. (1999) reported that inhalation of LPS at concentrations similar to those found in swine buildings (2.5 μg/m^3 air) did not affect the respiratory tract. Like in humans, LPS (≥5 mg/kg body weight) has been shown to induce the release of TNF-α and IL-1 in BAL fluid of pigs (Van Reeth et al., 2000).

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2.

AIMS OF THE THESIS

Aims of the thesis

Multi-factorial respiratory disease problems in young pigs are responsible for important financial losses in the swine industry. A variety of respiratory pathogens and adverse environmental conditions contribute to the development of these problems with porcine reproductive and respiratory syndrome virus (PRRSV) as an important factor. The general aims of the present thesis were to extend the knowledge on the pathogenesis of a PRRSV infection in the lungs of pigs, to examine if there exists a clinical synergy between PRRSV and lipopolysaccharide (LPS), and to evaluate the safety and efficacy of modified live PRRSV vaccines.

The specific aims can be defined as follows:

(1) To study a number of pathogenetic events in the lungs during a PRRSV infection in detail (virus replication, changes in the broncho-alveolar lavage (BAL) cell population and humoral immunity).

(2) To study the kinetics of apoptosis in the lungs and BAL cells during a PRRSV infection and to examine if correlations exist between virus replication, the presence of apoptosis and the production of cytokines.

(3) To study the clinical outcome of inoculations with virulent PRRSV followed by LPS and the effect of the timing and frequency of LPS administrations.

(4) To study the safety aspects of attenuated PRRSV strains using virological and clinical parameters.

(5) To evaluate the efficacy of commercially available modified live PRRSV vaccines using virological and clinical parameters of protection.

3.

PATHOGENESIS OF A PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION IN THE LUNGS OF PIGS

3.1.

EFFECT OF CELLULAR CHANGES AND ONSET OF HUMORAL IMMUNITY ON THE REPLICATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IN THE LUNGS OF PIGS

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Summary

Twenty-two four- to five-week-old gnotobiotic pigs were inoculated intranasally with $10^{6.0}$ $TCID_{50}$ of porcine reproductive and respiratory syndrome virus (PRRSV) (Lelystad virus) and euthanized at different time intervals post inoculation (PI). Broncho-alveolar lavage (BAL) cell populations were characterized, together with the pattern of virus replication and appearance of antibodies in the lungs. Total BAL cell numbers increased from 140 $x10^6$ at 5 days PI to 948 $x10^6$ at 25 days PI and remained at high levels till the end of the experiment. The number of monocytes/macrophages, as identified by monoclonal antibodies 74-22-15 and 41D3, increased 2- to 5-fold between 9 and 52 days PI with a maximum at 25 days PI. Flow cytometry showed that the population of differentiated macrophages was reduced between 9 and 20 days PI and that between the same time interval, both 74-22-15-positive and 41D3-negative cells, presumably monocytes, and 74-22-15- and 41D3-double negative cells, presumably non-phagocytes, entered the alveolar spaces. Virus replication was highest at 7 to 9 days PI, decreased slowly thereafter and was detected until 40 days PI. Anti-PRRSV antibodies were detected starting at 9 days PI but neutralizing antibodies were only demonstrated in one pig euthanized at 35 days and another at 52 days PI. The decrease of virus replication in the lungs from 9 days PI can be attributed to (i) shortage of susceptible differentiated macrophages, (ii) lack of susceptibility of the newly infiltrated monocytes and (iii) appearance of anti-PRRSV antibodies in the lungs. Neutralizing antibodies may contribute to the clearance of PRRSV from the lungs.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first observed in the United States in 1987 as a new viral disease of swine (Hill, 1990) and appeared in Europe in 1990 (Terpstra et al., 1991). Since then, the virus spread to all major swine-producing countries worldwide. The porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of the disease, was first isolated in the Netherlands in 1991 (Wensvoort et al., 1991) and subsequently in the United States in 1992 (Collins et al., 1992). A PRRSV infection in swine is clinically characterized by reproductive failure in sows and gilts and respiratory distress in young pigs. However, overt respiratory signs are difficult to reproduce and in most experimental studies, the infection is subclinical or a transient fever is the only clinical sign (Van Reeth et al., 1996). Based on the similarity in morphology, genomic organization, and strategy of gene expression, PRRSV has been classified as a member of the family of the *Arteriviridae* (Cavanagh, 1997). All viruses of this family have common features in so far that (i) cells of the monocyte/macrophage lineage are the primary or the only target cells *in vivo* and that (ii) they cause persistent infections in their respective hosts (Plagemann & Moennig, 1992).

PRRSV has a strong tropism for cells of the monocyte/macrophage lineage. However, replication of the virus in these cells is subject to several restrictions. It is confined to well-differentiated cells of the monocyte/macrophage lineage in lungs and lymphoid tissues and is not detected in progenitor cells such as bone marrow cells and peripheral blood monocytes (Duan et al., 1997b). This restricted cell tropism for PRRSV is partly due to the expression of the PRRSV receptor on the membrane of susceptible pulmonary alveolar macrophages (PAMs), which is not detectable on refractory peripheral blood monocytes (Duan et al., 1998b). Also, PRRSV replicates only at a certain stage of differentiation and maturation of the alveolar macrophages (Choi et al., 1994; Duan et al., 1997b). The complex effect of phenotype on the susceptibility of alveolar macrophages forms probably the basis for the low percentage of PRRSV antigen-positive broncho-alveolar lavage (BAL) cells after infection even in the period of the most extensive virus replication in the lungs (Mengeling et al., 1995; Duan et al., 1997a).

PRRSV is able to persist in different organs. It can be detected in lungs till 35 (Duan et al., 1997a) to 49 days after inoculation (Mengeling et al., 1995), in tonsils till 21 days after inoculation (Duan et al., 1997a), in semen till 92 days after inoculation (Christopher-Hennings et al., 1995) and in serum till 58 days after inoculation (Sur et al.,

1997) and persistence occurs despite the onset of a PRRSV-specific humoral and cell-mediated immune response. The mechanism of persistence is unknown.

The purpose of the present experiment was to study a number of pathogenetic events in the lungs during a PRRSV infection in gnotobiotic pigs in detail. The BAL cell population was examined, consisting of quantification of cells, determination of cell viability, morphological and phenotypical characterization of different subpopulations with specific attention to the population of cells of the monocyte/macrophage lineage and determination of presence of the PRRSV receptor and these events were correlated with virus replication (titration and quantification of viral antigen-positive cells) and appearance of neutralizing and non-neutralizing antibodies. These studies show that a single PRRSV infection causes multiple marked changes in the lungs even though clinical signs remain absent.

Materials and Methods

Virus strain. PRRSV (Lelystad virus) (Wensvoort et al., 1991) was used in the present study. Virus used for inoculation was at the fifth passage in PAMs, which had been obtained from four- to six-week-old gnotobiotic pigs.

Pigs and inoculation. A total of twenty-nine caesarean-derived colostrum-deprived (CDCD) pigs were used. They were housed in isolation facilities. Twenty-two pigs were inoculated intranasally at the age of 4 to 5 weeks with $10^{6.0}$ $TCID_{50}$ Lelystad virus in 3 ml phosphate-buffered saline (PBS) (1.5 ml in each nostril). The remaining seven pigs were left non-inoculated and served as negative controls. One to three of the PRRSV-inoculated pigs were euthanized at 1 (n=1), 3 (n=2), 5 (n=2), 7 (n=3), 9 (n=3), 14 (n=3), 20 (n=1), 25 (n=2), 30 (n=1), 35 (n=2), 40 (n=1), and 52 (n=1) days post inoculation (PI) by intraperitoneal injection with an overdose of barbiturates (Natriumpentobarbital® 20%, IC KELA).

The control pigs were euthanized at 4 (n=2), 5 (n=1), 6 (n=1), 8 (n=2), and 10 (n=1) weeks of age. The right lung was used for broncho-alveolar lavage and samples from the left apical, cardiac and diaphragmatic lung lobes were collected for virological examinations (virus titration and quantification of viral antigen-positive cells).

Broncho-alveolar lavage (BAL) cell analysis. The right lung was lavaged with 60 to 120 ml Dulbecco's PBS without Ca^{2+} and Mg^{2+} via an 18-gauge blunt needle inserted

through the trachea. The left main bronchus was cross-clamped to prevent lung lavage fluid from entering the left lung. About 75-90% of the initial volume of the lavage fluid was recovered. The BAL fluid was centrifuged (400xg, 10 minutes, 4°C) to separate the cells and the cell-free lavage fluid.

Fractions of the cell-free lavage fluid were either stored at –70°C until virus titration on PAMs or concentrated 10 times by dialysis against a 20% w/v solution of polyethylene glycol (MW 20,000) and cleared of residual virus by ultracentrifugation at 100,000xg (Van Reeth et al., 1999) for determination of anti-PRRSV antibodies.

Cell pellets were resuspended in PBS and the total number of BAL cells was determined. Cell viability was assessed using trypan blue dye exclusion. Cytocentrifuge preparations of BAL cells were made by centrifuging at 140xg for 5 minutes. One preparation was stained with DiffQuick (Baxter) to determine the percentage of mononuclear cells and neutrophils, another was fixed in acetone for 20 minutes at –20°C to determine the percentage of viral antigen-positive cells using a streptavidin-biotin immunofluorescence technique. Cells were first incubated with a pool of monoclonal antibodies (MAbs) against the PRRSV nucleocapsid protein (dilution 1/100 of WBE1 and WBE4-6) (Drew et al., 1995), subsequently with 1/100 biotinylated sheep anti-mouse antibodies (Amersham), and finally with 1/100 streptavidin-fluorescein isothiocyanate (FITC) (Amersham). Finally, cells were washed, mounted in a glycerin-PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazobicyclo-2.2.2-octane (DABCO) (Janssen Chimica) and viral antigen-positive cells were counted by fluorescence microscopy (Leica DM RBE, Wild Leitz). The specificity was confirmed by absence of fluorescence in BAL cells of uninoculated control pigs.

Flow cytometric analyses of the BAL cells were done with a Becton-Dickinson FACSCalibur™ equipped with a 15mW aircooled argon ion laser and interfaced to a Macintosh Quadra 650 computer (Apple Computer) using BD Cellquest software. Three parameters were stored for further analysis: forward light scattering (FSC), sideward light scattering (SSC) and green fluorescence (FL1). At least 10,000 cells were analysed for each sample.

For the phenotypic identification of neutrophils and cells of the monocyte/macrophage lineage, MAb 74-22-15 (Pescovitz et al., 1984) was used. The percentage of cells of the monocyte/macrophage lineage was determined by subtracting the percentage of neutrophils, as determined by DiffQuick, from the 74-22-15-positive cells. The percentage of BAL cells with expression of the PRRSV receptor on their cell membrane

was determined using MAb 41D3 (Duan et al., 1998a). The reactivity of both monoclonal antibodies against the specific cell surface determinants was flow cytometrically evaluated by an indirect immunofluorescence technique. To determine the number of 74-22-15- and 41D3-positive BAL cells, 5 $x10^6$ cells were incubated with 1/300 74-22-15 or 1/1000 41D3, respectively for 1 hour at 4°C. Subsequently, the BAL cells were incubated with 1/30 FITC-labelled goat anti-mouse IgG (Molecular Probes) for 1 hour at 4°C. Three washings were done with cold PBS before and after each incubation. BAL cells which were only incubated with FITC-labelled goat anti-mouse IgG were included as controls.

Virological examinations of lung tissue and BAL fluid. Twenty percent suspensions of lung tissue were made with cold PBS. The suspensions were clarified by centrifugation. Fifty µl of tenfold serial dilutions of the supernatants of the lung suspensions and of the cell-free lavage fluids were inoculated on one-day cultivated PAMs, which were obtained from four- to six-week-old pigs from PRRSV-negative farms. After incubation for 1 hour at 37°C, the samples were replaced by medium. After 72 hours at 37°C, the PAMs were washed once with PBS and further stained using an immunoperoxidase monolayer assay (IPMA) as described by Wensvoort et al. (1991).

Tissue samples from the lungs were embedded in methylcellulose medium and frozen at –70°C. Cryostat sections (5-8 µm) were made and fixed in acetone for 20 minutes at –20°C. A streptavidin-biotin immunofluorescence technique, similar as described for BAL cells, was used to count and localize the viral antigen-positive cells in lung tissue.

Serological examinations. Anti-PRRSV antibody titres were determined in sera and BAL fluids using an IPMA. IPMAs with MARC-145 cells were set up to determine the immunoglobulin (Ig) isotypes of the PRRSV-specific antibodies. Briefly, MARC-145 cells were seeded in 96-well cell culture plates, inoculated with 50 µl of a Belgian isolate of PRRSV (94V360) and incubated for 18 hours (37°C, 5% CO_2). Then, the culture medium was removed, cells were washed in PBS and dried at 37°C for 1 hour. The plates were kept at –70°C until use. Plates were thawed and then fixed in 4% paraformaldehyde for 10 minutes. Paraformaldehyde was removed, the cells were washed twice with PBS and a solution of 1% hydrogen peroxide in methanol was added. Plates were washed twice with PBS and serial four-fold dilutions of the sera and BAL

fluids were added. Sera and BAL fluids were incubated for 1 hour at 37°C. Plates were washed three times with PBS plus 1% Tween 80 and 50 µl of 1/10 mouse anti-swine IgG1, 1/10 mouse anti-swine IgG2, 1/100 mouse anti-swine IgM, and 1/160 mouse anti-swine IgA (Van Zaane & Hulst, 1987), respectively were added per well and incubated at 37°C for 1 hour. Plates were washed three times and 50 µl of 1/3000 goat anti-mouse antibodies conjugated with peroxidase (Dako) were added per well and incubated at 37°C for 1 hour. Plates were washed three times and 50 µl of a substrate solution of 3-amino-9-ethylcarbazole in 0.05 M acetate buffer, pH 5 with 0.05% H_2O_2 were added to each well and incubated at room temperature for 20 minutes. Then, the reaction was blocked by replacing the substrate by acetate buffer and the result was examined with a microscope.

Neutralizing antibodies were determined in sera and BAL fluids using a virus neutralization test on MARC-145 cells as described by Swenson et al. (1994). A MARC-145-adapted Lelystad strain was used in this assay.

Results

BAL cell analysis. Total BAL cell numbers of non-inoculated control pigs ranged between 114 and 383 $x10^6$. BAL cells consisted of 97-98% of cells of the monocyte/macrophage lineage, 1% of neutrophils and 1-2% of non-phagocytes, presumably lymphocytes (BAL cells minus 74-22-15-positive cells). Cell viability of BAL cells ranged between 93 and 98%. BAL cell populations in PRRSV-infected pigs are shown in Figure 1. Their number and composition were similar to those of the non-inoculated control pigs during the first 5 days PI. Mean BAL cell numbers increased from 140 $x10^6$ at 5 days PI to 948 $x10^6$ at 25 days PI and then remained at high levels till the end of the experiment with numbers ranging between 642 and 782 $x10^6$. The absolute number of cells of the monocyte/macrophage lineage, as identified by MAb 74-22-15, increased 2- to 5-fold between 9 and 52 days PI with a maximum at 25 days PI. BAL cells consisted of 55-92% of cells of the monocyte/macrophage lineage, 1-15% of neutrophils (33% in one pig euthanized at 9 days PI) and 6-31% of non-phagocytes. Cell viability of BAL cells ranged between 84 and 98%.

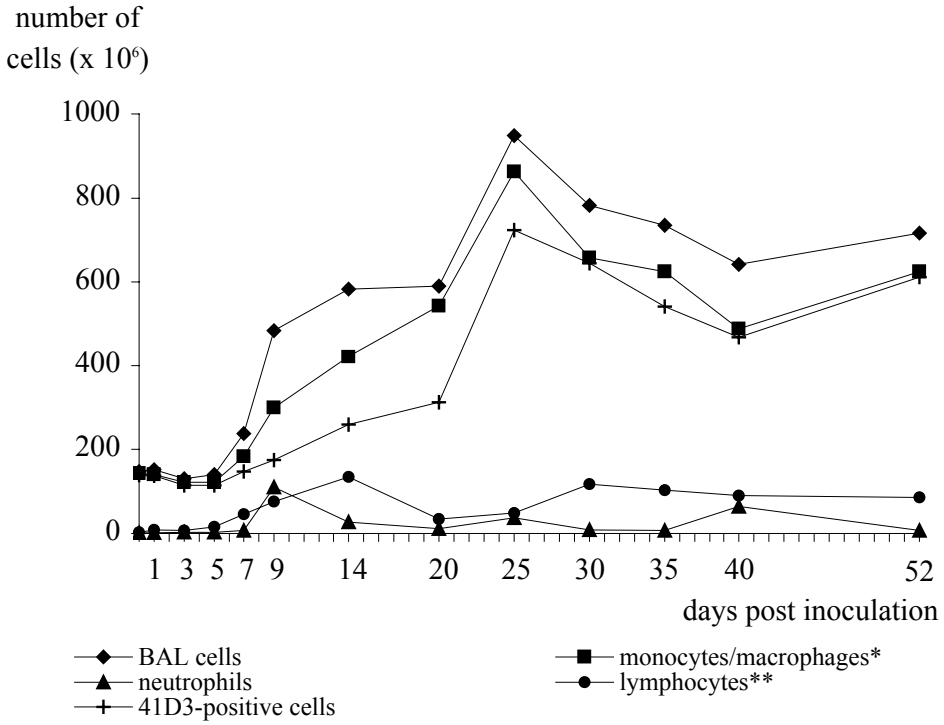

Fig. 1. Quantification and characterization of broncho-alveolar lavage (BAL) cells (x10^6/lung half) throughout a PRRSV infection.

*Cells of the monocyte/macrophage lineage = 74-22-15-positive cells – neutrophils; **Lymphocytes = BAL cells – 74-22-15-positive cells.

In the non-inoculated control pigs, the percentage of BAL cells with the PRRSV receptor (41D3-positive cells) varied between 93 and 95%. In PRRSV-infected pigs, their percentage was similar to that of the controls during the first 3 days PI. At 5, 7, 9, 14, 20, and 25 days PI, respectively, 41D3-positive BAL cells represented 83 ± 1, 63 ± 5, 38 ± 14, 41 ± 9, 53 ± 0, and 75 ± 4% of total BAL cells. From 30 days PI, the percentage of 41D3-positive BAL cells reached the percentage of cells of the monocyte/macrophage lineage.

The different patterns of scattering light characteristics [cell size (FSC) and granularity (SSC)] of the BAL cell population throughout a PRRSV infection are presented in Figure 2 (dot-plot diagrams).

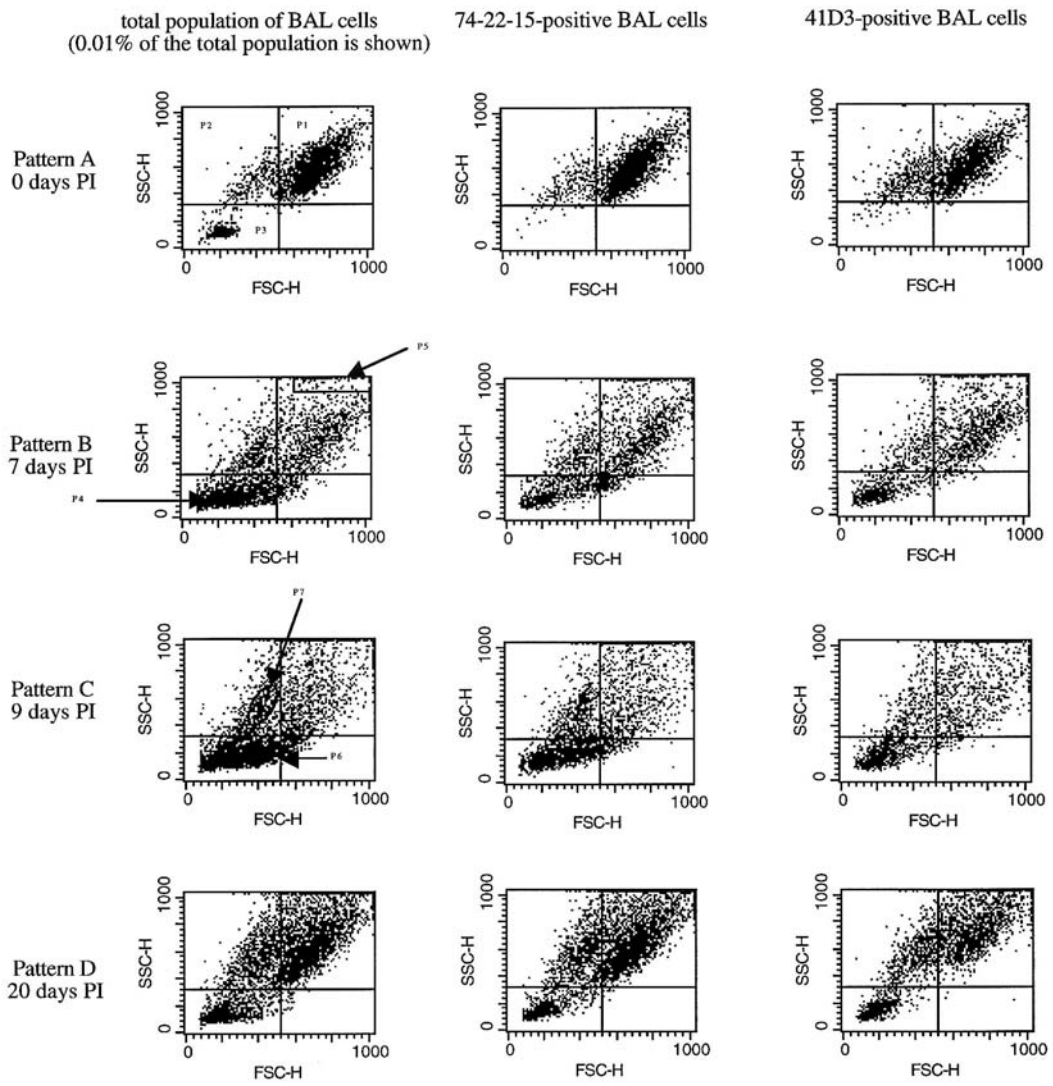

Fig. 2. Different patterns of light-scattering characteristics [FSC (cell size) and SSC (granularity)] of the broncho-alveolar cell population throughout a PRRSV infection.

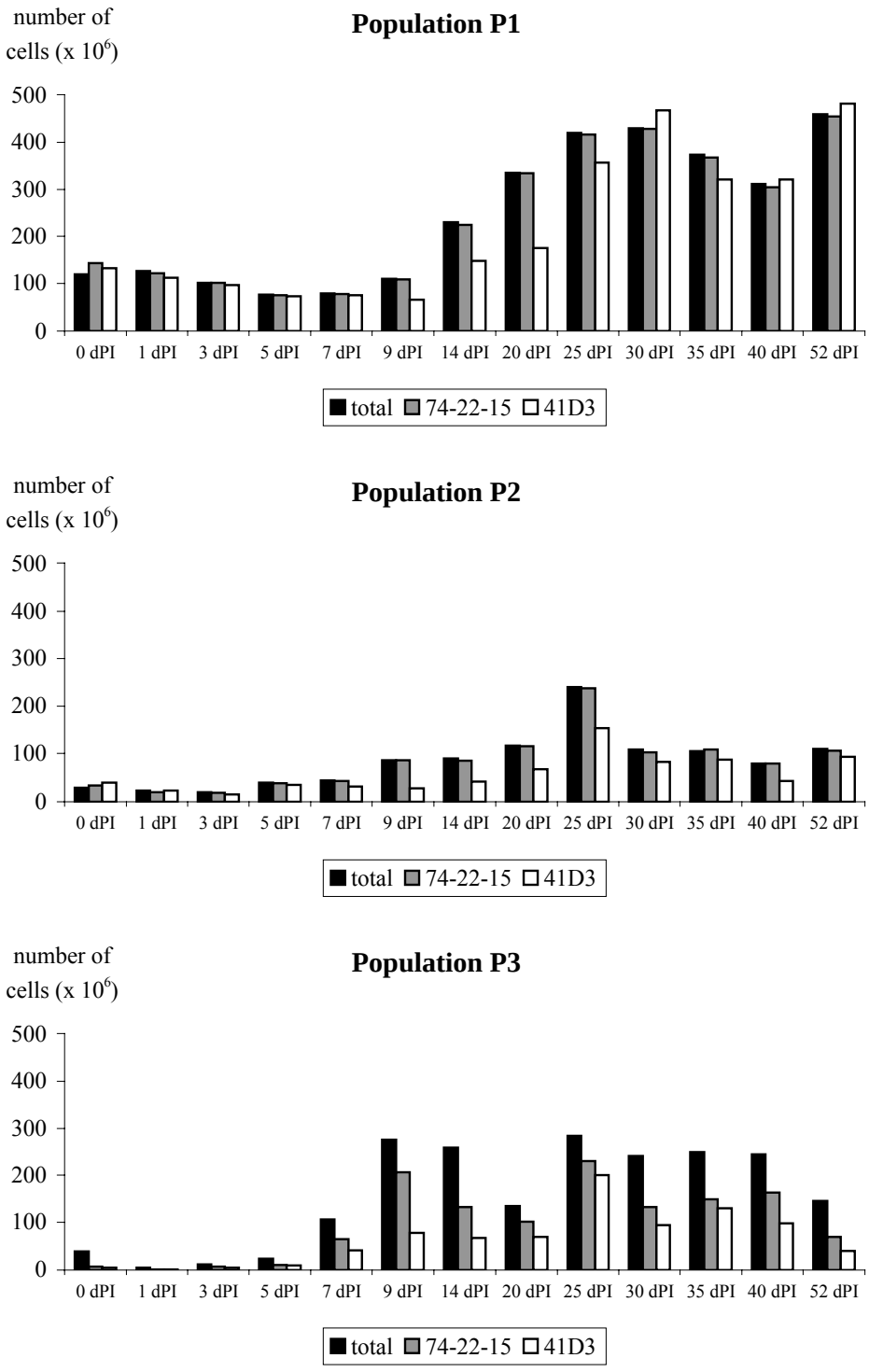

Fig. 3. Changes in the number of cells of broncho-alveolar lavage (BAL) cell populations P1, P2 and P3 (x 10^6/lung half) throughout a PRRSV infection.

In the non-inoculated control pigs, the majority of BAL cells were flow cytometrically recognized as large (high FSC value) (population P1) and small (low FSC value) (population P2) cells with a strong granularity (high SSC value). Based on their scattering light properties and their surface expression of a specific monocyte/granulocyte marker (recognized by MAb 74-22-15) and of the PRRSV receptor (recognized by MAb 41D3), these two cell populations were characterized as cells of the monocyte/macrophage lineage. The particles with the lowest scattering light properties (population P3) were characterized as non-phagocytes (74-22-15- and 41D3-double negative cells) and fragments of cells of the monocyte/macrophage lineage (74-22-15-single positive and 74-22-15- and 41D3-double positive). The evolution of the number of cells of BAL cell populations P1, P2 and P3 throughout the infection are presented in Figure 3. In PRRSV-infected pigs, the scattering light characteristics of the BAL cell population were similar to those of the non-inoculated control pigs during the first 5 days PI. Between 9 and 20 days PI, the number of 41D3-positive cells of the monocyte/macrophage lineage in populations P1 and P2 was reduced. From 7 days PI, an increase of very small particles with low scattering light properties was observed (population P4) and these particles stained positive for both 74-22-15 and 41D3. From 7 till 20 days PI, very large and strongly granulated cells were present in the BAL fluid. They exceeded the maximal FSC and SSC values shown on the dot-plot diagrams in Figure 2 (population P5). Between the same time interval, populations of small cells were entering the alveolar spaces. The slightly granulated (low SSC value) cells (population P6) were characterized as, on the one hand 74-22-15-positive and 41D3-negative cells, representing probably monocytes and, on the other hand 74-22-15- and 41D3-double negative cells, representing probably non-phagocytes (presumably lymphocytes). The strongly granulated cells (high SSC value) were only observed at 9 and 14 days PI (population P7). These cells were also 74-22-15-positive and 41D3-negative. From 25 till 52 days PI, the majority of BAL cells were recognized as 74-22-15- and 41D3-positive cells of the monocyte/macrophage lineage and 74-22-15- and 41D3-double negative cells, presumably lymphocytes.

Virological examinations of lung tissue and BAL fluid. The results of virus titrations of lung tissue and BAL fluid are presented in Figure 4.

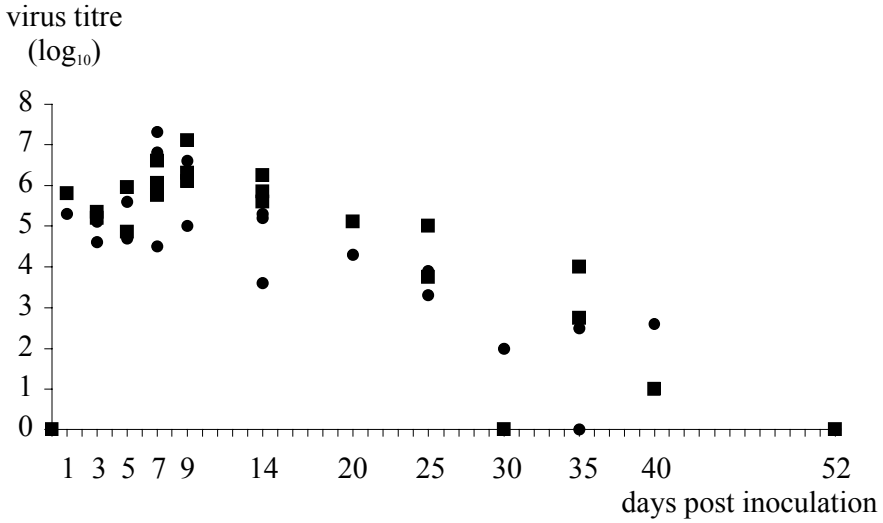

Fig. 4. Course of PRRSV titres in lung tissue (log_{10} $TCID_{50}$/gram) and broncho-alveolar lavage (BAL) fluid (log_{10} $TCID_{50}$/ml) throughout a PRRSV infection (■: lung tissue; ●: BAL fluid).

All non-inoculated control pigs were negative for PRRSV. In the inoculated pigs, PRRSV titres of apical, cardiac and diaphragmatic lung lobes were similar and the pattern of virus titres in lung tissue resembled that in BAL fluid. The highest virus titres were reached in BAL fluid at 7 days PI ($10^{7.3}$ $TCID_{50}$/ml) and in lung tissue at 9 days PI ($10^{7.1}$ $TCID_{50}$/gram). Afterwards, virus titres decreased slowly during the next five weeks. Virus was not detected in the pig euthanized at 52 days PI.

Viral antigen-positive BAL cells were first observed at 1 day PI, increased to a maximum of 3% at 9 days PI, decreased to 0.5% at 14 days PI and remained at levels of 0.1-0.2% until 40 days PI. The quantification of viral antigen-positive cells and/or foci in lung tissue throughout a PRRSV infection is presented in Figure 5. Single viral antigen-positive cells were observed from 3 until 35 days PI with a maximal number of 45 cells/100 mm^2 lung tissue at 7 days PI. Viral antigen-positive foci were defined as areas in lung tissue consisting of groups of viral antigen-positive cells and cellular debris. Viral antigen-positive foci were found from 3 until 14 days PI with a maximal number of 37 foci/100 mm^2 lung tissue at 9 days PI. The intact viral antigen-positive cells in lung tissue and BAL fluid were morphologically recognized as macrophage-like cells. Viral

antigen-positive cells were not observed in lung tissue and BAL fluid of non-inoculated control pigs.

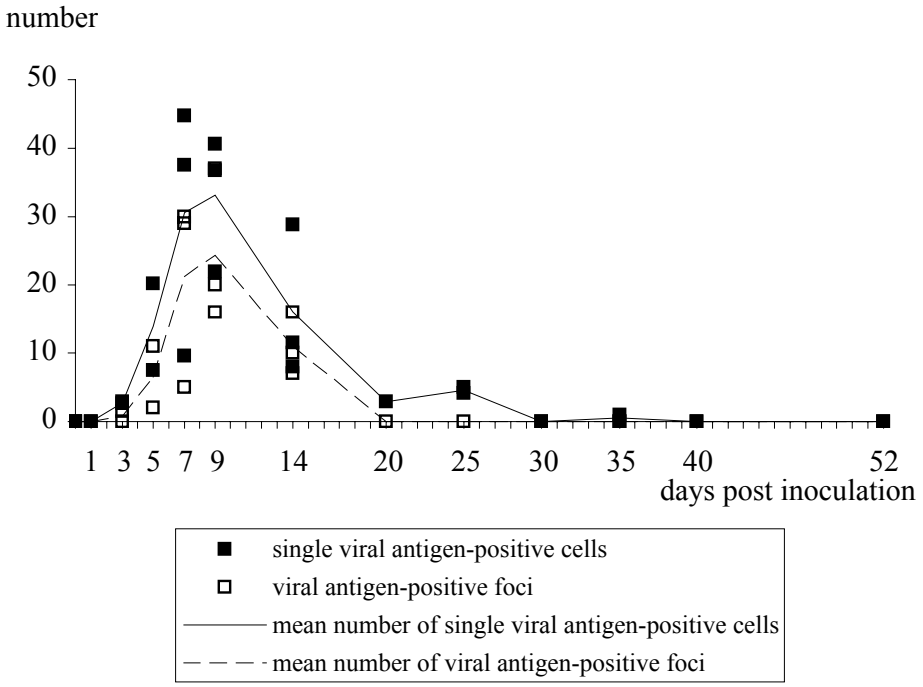

Fig. 5. Quantification of single viral antigen-positive cells and viral antigen-positive foci in lung tissue (/100 mm^2) throughout a PRRSV infection.

Antibodies in sera and BAL fluids. The antibody titres against PRRSV in sera and BAL fluids of PRRSV-inoculated pigs are presented in Figure 6 (on a logarithmic scale). PRRSV-specific antibodies in sera and BAL fluids were first detectable by the IPMA at 9 days PI. IPMA titres in serum rose to 10,240 ($2^{13.3}$) at 20 days PI, whereas IPMA titres in BAL fluid rose to 2560 ($2^{11.3}$) at 25 days PI. The distribution of the immunoglobulin isotypes of PRRSV-specific antibodies in sera and BAL fluids throughout the infection is presented in Figure 7 (on a logarithmic scale).

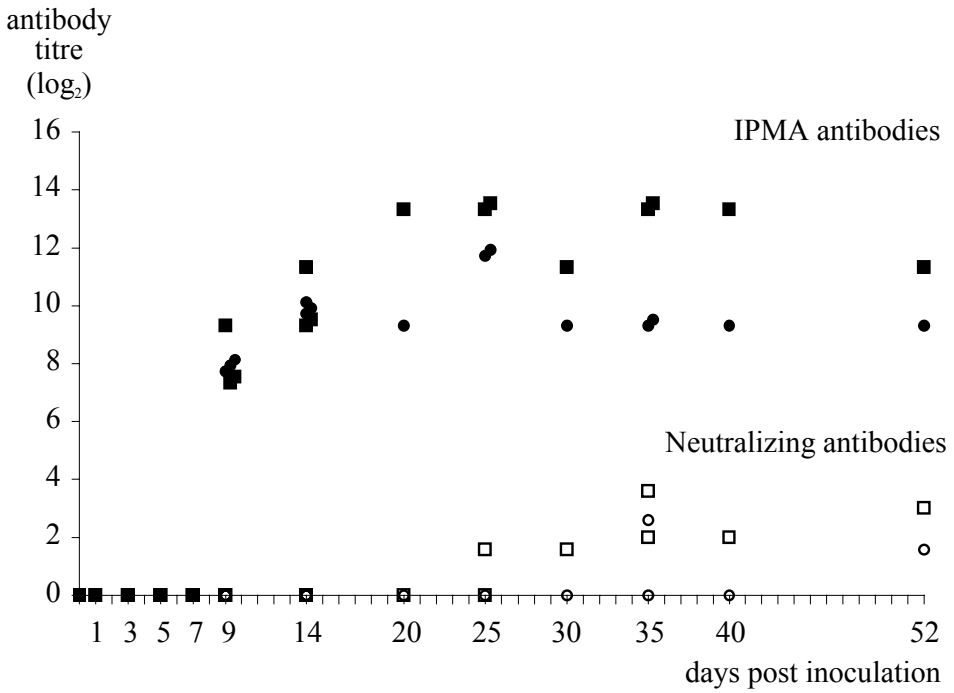

Fig. 6. Antibody titres against PRRSV in sera (squares) and broncho-alveolar lavage (BAL) fluids (bullets) of PRRSV-inoculated pigs.

The first detectable IPMA antibodies in sera and BAL fluids of PRRSV-inoculated pigs were antibodies of the IgM and IgG isotype (IgG1 subclass). IgM antibodies in sera and BAL fluids were detected only at 9 and 14 days PI, whereas IgG antibodies were detected until 52 days PI. IgG1 IPMA titres in sera and BAL fluids followed the curve of total IPMA titres. The curve of IgG2 IPMA titres in sera and BAL fluids was similar to that of IgG1 IPMA titres, but was at a lower level. IgA antibodies in sera were detected starting from 14 days PI, increased to a maximum of 1280 ($2^{10.3}$) at 25 days PI and were detected until 35 days PI. IgA antibodies in BAL fluids were highest at 14 days PI ($2^{6.0}$) and were detected until 35 days PI. Neutralizing antibodies in sera were detected from 25 days PI. The titres remained at a low level ($2^{1\text{-}3.6}$) until the end of the study. Neutralizing antibodies in BAL fluids were only detected in two pigs, one euthanized at 35 days and one at 52 days PI.

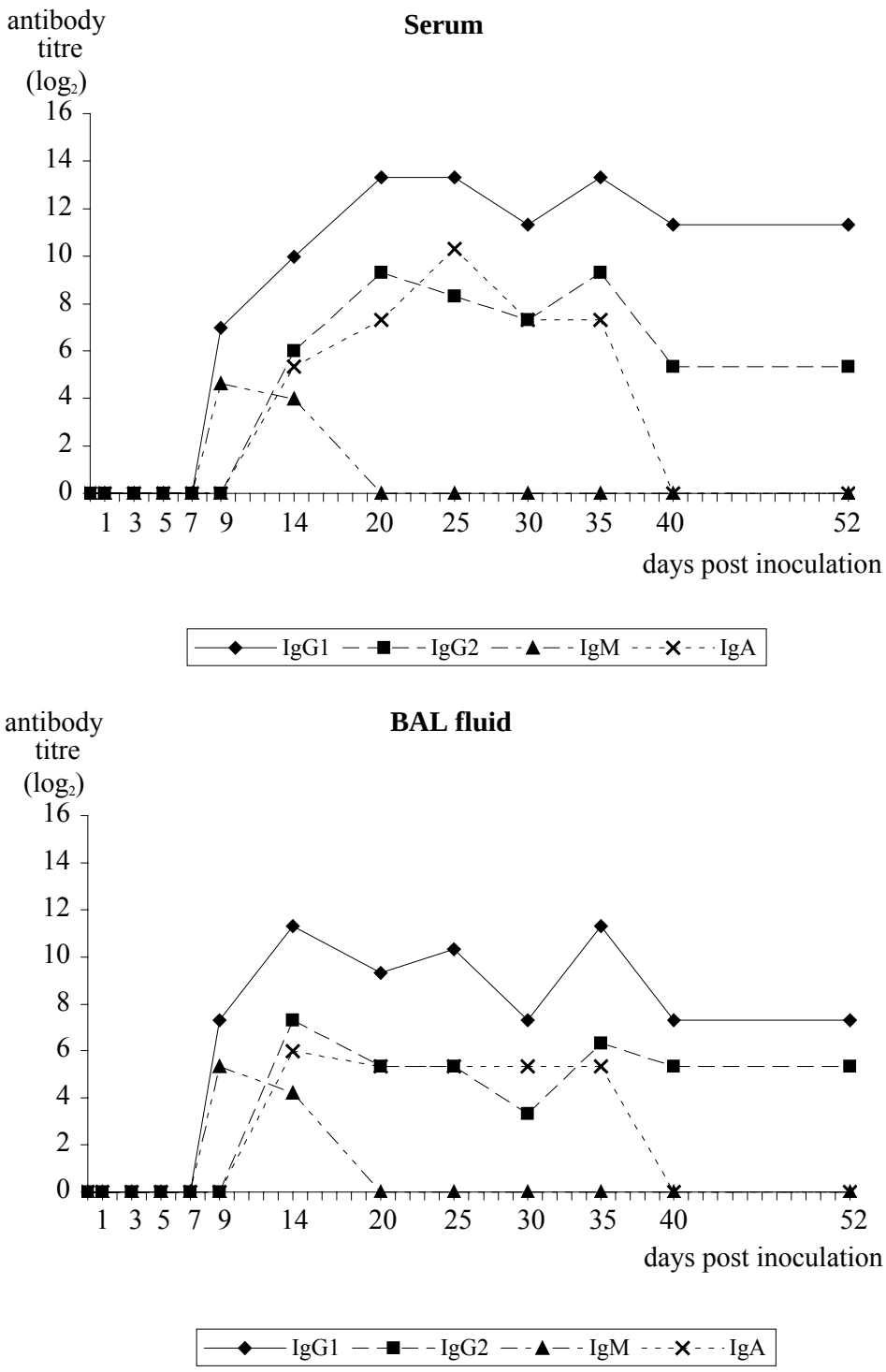

Fig. 7. Distribution of immunoglobulin isotypes of PRRSV-specific antibodies in sera and broncho-alveolar lavage (BAL) fluids of PRRSV-inoculated pigs.

Discussion

The present study showed clear relations between PRRSV replication, morphological and phenotypical changes in the broncho-alveolar lavage (BAL) cells, and onset and presence of non-neutralizing and neutralizing antibodies.

The most striking morphological and phenotypical changes in the BAL cell population consist of (i) the reduction of the population of susceptible well-differentiated macrophages and (ii) the massive influx of both 74-22-15-positive and 41D3-negative cells of the monocyte/macrophage lineage, probably monocytes, and 74-22-15- and 41D3-double negative cells, probably non-phagocytes.

Between 9 and 20 days PI, a reduction of the population of 41D3-positive cells of the monocyte/macrophage lineage was observed. The reduction of this cell population is probably caused by a combination of cell lysis due to virus replication and apoptosis. The highest virus titres and numbers of viral antigen-positive cells in lungs and BAL fluids were indeed detected at 7 to 9 days PI and apoptosis in the lungs between 1 and 10 days PI was earlier demonstrated by Sirinarumitr et al. (1998) and Sur et al. (1998). These authors reported that apoptotic cells were pulmonary alveolar and intravascular macrophages and mononuclear cells in the alveolar septa and showed that they were more abundant than viral antigen-positive cells. The increased number of very small and slightly granulated particles, which stained positive for both 74-22-15 and 41D3, in the BAL cell population from 7 days PI on, may represent an increase of lysed infected macrophages and apoptotic bodies from macrophages in apoptosis. The very large and strongly granulated cells, found in the BAL cell population from 7 till 20 days PI, may represent strongly activated macrophages phagocytizing the apoptotic bodies. The biological significance of apoptosis in the pathogenesis of a PRRSV infection remains to be clarified.

Throughout the PRRSV infection in the lungs of gnotobiotic pigs, the total number of BAL cells continuously increased from 5 days PI till 25 days PI mainly due to an influx of both 74-22-15-positive and 41D3-negative cells, probably monocytes, and 74-22-15- and 41D3-double negative cells, probably non-phagocytes (lymphocytes). This observation is supported by the flow cytometric analysis of the BAL cell population, evaluating the evolution of size (FSC), granularity (SSC) and presence of markers on the cell surface throughout the infection. The mechanism by which PRRSV induces the specific influx of monocytes is not yet known, but Van Reeth et al. (1999) suggested that

chemotactic cytokines produced by PRRSV-infected macrophages mediate the influx of new cells of the monocyte/macrophage lineage.

Despite the continuous increase of the number of cells of the monocyte/macrophage lineage from 9 till 25 days PI, the number of viral antigen-positive cells in lung tissue and BAL fluid decreased from 9 days PI. This is probably the result of (i) shortage of susceptible well-differentiated lung macrophages (see above), (ii) lack of susceptibility of the newly infiltrated blood monocytes (Duan et al., 1997b) and (iii) appearance of anti-PRRSV antibodies in the lungs. It is remarkable that viral antigen-positive cells and cellular debris were mainly localized in foci in lung tissue until 14 days PI and that, thereafter, only single viral antigen-positive cells were observed. It is possible that infected cells in foci are destroyed by antibody-dependent cell lysis. Single viral antigen-positive cells, morphologically recognized as macrophage-like cells, were observed until 35 and 40 days PI in lung tissue and BAL fluid, respectively. These single viral antigen-positive cells were the source of the virus detected in lung tissue and BAL fluid until 40 days PI. Why these single viral antigen-positive cells are able to persist despite the presence of the humoral immunity is not known, but may be attributed to either the cell or the virus. Therefore, a full phenotypical characterization of this specific subpopulation of cells will be performed. An alternative explanation may be the appearance of so-called quasispecies throughout the PRRSV infection. A recent study of Rowland et al. (1999) revealed the emergence of a distinct PRRSV subpopulation during infection of pigs, identified by a single amino acid change in the ectodomain of glycoprotein 5. Studies using lactate dehydrogenase-elevating virus of mice, another member of the family of the *Arteriviridae*, demonstrated how a small number of mutations in the ectodomain of the open reading frame (ORF) 5 protein can alter the cell tropism and the interaction of the virus with neutralizing antibodies, favouring persistence of the virus in its host (Plagemann, 1996 ; Chen et al., 1997).

Immunoglobulin M, A, G1 and G2 (IgM, IgA, IgG1, and IgG2) antibodies were all involved in the specific humoral immune response to PRRSV. IgM antibodies appear rather late during a PRRSV infection, which is in contrast with infections with other respiratory viruses such as influenza virus (Lee et al., 1995) and Aujeszky's disease virus (Rodak et al., 1987), where IgM antibodies were detected as early as 3 and 7 days PI, respectively. The kinetics of the isotypes of anti-PRRSV antibodies in BAL fluids were similar to those in sera, indicating that these antibodies are the result of a leakage from systemic antibodies.

Clearance of PRRSV from the lungs coincided with the appearance of neutralizing antibodies in sera and BAL fluids. However, since low amounts of PRRSV remain in the lungs in spite of the presence of neutralizing antibodies in sera and BAL fluids, other immune factors or mechanisms, such as cell-mediated immunity, are probably involved in the complete elimination of the virus at this site. Why neutralizing antibodies appear so late in infection and remain at rather low levels (titres ranging between 2 and 12) is not yet known.

Based on the results of the present study, a hypothetical model of the series of events in the pathogenesis of a PRRSV infection in the lungs of gnotobiotic pigs can be made. After inoculation, primary replication of the virus takes place in well-differentiated lung macrophages. Subsequently, a reduction of the resident macrophages takes place, which is accompanied with an influx of new cells of the monocyte/macrophage lineage in the lungs, which are initially refractory to PRRSV. Lack of susceptible cells and the appearance of PRRSV-specific antibodies cause a decrease in the number of viral antigen-positive cells starting at 9 days PI. Nevertheless, a low number of single viral antigen-positive cells persists in the lungs for several weeks. Clearance of PRRSV from the lungs coincides with the appearance of specific neutralizing antibodies. Most likely, other mechanisms, such as cell-mediated immunity, are necesarry for a complete elimination.

Acknowledgements

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3.2.

APOPTOSIS IN THE LUNGS OF PIGS INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND ASSOCIATIONS WITH THE PRODUCTION OF APOPTOGENIC CYTOKINES

Veterinary Research, conditionally accepted

Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K. and Pensaert, M.

Summary

Apoptosis was studied in the lungs of pigs during an infection with a European strain of porcine reproductive and respiratory syndrome virus (PRRSV) with the purpose to find its potential role in the pathogenesis. Additionally, it was examined if cytokines are involved in the induction of apoptosis. Twenty-two four- to five-week-old gnotobiotic pigs were inoculated intranasally with $10^{6.0}$ $TCID_{50}$ of Lelystad virus and euthanized between 1 and 52 days post inoculation (PI). Lungs and broncho-alveolar lavage (BAL) cells were assessed both for virus replication and apoptosis; BAL fluids were examined for interleukin (IL)-1, tumour necrosis factor-alpha (TNF-α) and IL-10. Double-labelings were conducted to determine the relation between virus replication and apoptosis and for phenotypical identification of the apoptotic cells. Apoptosis occurred in both infected and non-infected cells. The percentages of infected cells, which were apoptotic, ranged between 9 and 39% in lung tissue and between 13 and 30% in BAL cells. The majority of apoptotic cells (> 99%) were non-infected. Non-infected apoptotic cells in lung tissue were predominantly monocytes/macrophages, whereas these in the broncho-alveolar spaces were predominantly lymphocytes. The peak of apoptosis in lung tissue at 14 days PI was preceded by a peak in IL-1 and IL-10 production in BAL fluids at 9 days PI, suggesting a possible role of these cytokines in the induction of apoptosis in non-infected interstitial monocytes/macrophages. However, the latter hypothesis was not confirmed by *in vitro* studies, since blood monocytes or alveolar macrophages did not undergo apoptosis after treatment with recombinant porcine IL-1 or IL-10. Apoptosis of non-infected interstitial monocytes/macrophages seems to be a homeostatic mechanism that serves to regulate the number of monocytes/macrophages during the massive influx of monocytes into the lungs of PRRSV-infected pigs.

Introduction

Apoptosis is a physiological mechanism of cell death important for normal cellular turnover that is characterized by internucleosomal DNA degradation and pronounced morphological changes (Wyllie et al., 1980). It also occurs during viral infections and there is mounting evidence that it can contribute directly to the viral pathogenesis (Collins, 1995).

Recently, it has been demonstrated that porcine reproductive and respiratory syndrome virus (PRRSV) induces apoptosis in lungs (Sirinarumitr et al., 1998; Sur et al., 1998; Labarque et al., 2001; Choi & Chae, 2002). On a morphological basis, the apoptotic cells were assumed to be alveolar and pulmonary intravascular macrophages and mononuclear cells in the alveolar septa. Double-labeling experiments indicated that most apoptotic cells were non-infected (Sirinarumitr et al., 1998; Sur et al., 1998).

An infection of lungs with PRRSV has some peculiar cellular features that may be attributed to apoptosis. Firstly, it is characterized by changes in the population of broncho-alveolar monocytes/macrophages, consisting of a reduction of the population of well-differentiated macrophages and an influx of new monocytes (Labarque et al., 2000). Since apoptosis is an important mechanism of cell population control in normal tissue homeostasis, it may be involved in the regulation of the number of monocytes/macrophages during this massive influx of new monocytes. Secondly, PRRSV causes only minimal lung inflammation (Pol et al., 1991) and neutrophil infiltration (Van Reeth et al., 1999; Labarque et al., 2000), which also may be attributed to the involvement of apoptotic processes, since apoptotic bodies are phagocytised by resident macrophages without provoking an inflammatory response.

Viral glycoprotein GP_5 has been shown to be involved in apoptosis of the PRRSV-infected cell (Suárez et al., 1996). However, the mechanism of apoptosis in the non-infected cell is unknown. It has been demonstrated that cytokines, such as tumour necrosis factor-alpha (TNF-α) (Larrick & Wright, 1990), interferon-alpha (IFN-α) (Suzuki et al., 1996), interleukin (IL)-1 (Dunger et al., 1996; Castigli et al., 2000) and IL-10 (Estaquier & Ameisen, 1997; Wang et al., 2001) can induce apoptosis. A PRRSV infection generally fails to induce substantial amounts of IFN-α (Albina et al., 1998; Trebichavsky & Valicek, 1998; Van Reeth et al., 1999). This argues against a possible role of this cytokine in PRRSV-induced apoptosis. IL-1, however, is produced to high

levels in the lungs of PRRSV-infected pigs from 3 till 10 days after inoculation (Van Reeth et al., 1999) and a weak TNF-α production was demonstrated at 12 and 13 days after inoculation (Van Reeth, 1998). Further, the induction of IL-10 mRNA in alveolar macrophages has been reported in PRRSV-infected pigs (Thanawongnuwech et al., 2000).

In the present study, the kinetics of apoptosis both in the lungs and in broncho-alveolar lavage (BAL) cells were investigated in gnotobiotic pigs upon infection with a European strain of PRRSV, with the purpose to have a better understanding of its potential role in the pathogenesis. Further, it was examined if IL-1, TNF-α and IL-10 may be involved in the induction of apoptosis during a PRRSV infection.

Materials and Methods

Virus strain. A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV (Wensvoort et al., 1991) was used.

Pigs and experimental design. A total of twenty-nine caesarean-derived colostrum-deprived (CDCD) pigs were used. They were housed in isolation facilities. Twenty-two pigs were inoculated intranasally at the age of 4 to 5 weeks with $10^{6.0}$ $TCID_{50}$ of Lelystad virus in 3 ml phosphate-buffered saline (PBS) (1.5 ml in each nostril). The remaining seven pigs were left non-inoculated and served as negative controls. One to three of the PRRSV-inoculated pigs were euthanized at 1 (n=1), 3 (n=2), 5 (n=2), 7 (n=3), 9 (n=3), 14 (n=3), 20 (n=1), 25 (n=2), 30 (n=1), 35 (n=2), 40 (n=1), and 52 (n=1) days post inoculation (PI) by intraperitoneal injection with an overdose of barbiturates (Natriumpentobarbital® 20%, IC KELA). The control pigs were euthanized at 4 (n=2), 5 (n=1), 6 (n=1), 8 (n=2), and 10 (n=1) weeks of age.

The right lung was used for broncho-alveolar lavage and samples from the left apical, cardiac and diaphragmatic lung lobes were collected for quantification of infected cells and detection of apoptosis.

Collection of samples. The right lung was lavaged using a previously described method (Van Reeth et al., 1999). The BAL fluid was centrifuged (400xg, 10 minutes, 4°C) to separate cells and cell-free lavage fluid.

Cytocentrifuge preparations of BAL cells were made by centrifuging at 140xg for 5 minutes. Preparations were fixed in acetone for 20 minutes at –20°C for quantification of infected cells, or in 4% paraformaldehyde for 10 minutes at room temperature for detection of apoptosis.

Cell-free BAL fluids were concentrated 10 times by dialysis against a 20% w/v solution of polyethylene glycol (MW 20,000), cleared of residual virus by ultra-centrifugation at 100,000xg (Van Reeth et al., 1999), aliquoted and stored at –70°C, until analysis in cytokine bioassays (IL-1, TNF-α) or cytokine ELISA (IL-10).

Tissue samples from the left lung lobes were embedded in methylcellulose medium and frozen at –70°C. Cryostat sections (5 to 8 μm) were made and fixed in acetone for 20 minutes at –20°C for quantification of infected cells, or in 4% paraformaldehyde for 10 minutes at room temperature for detection of apoptosis.

Virological examinations. PRRSV-infected cells in lung tissue and BAL cells were quantified using a streptavidin-biotin immunofluorescence technique (Labarque et al., 2000).

Detection of apoptosis. Cytocentrifuge preparations of BAL cells and cryostat sections of lung tissue were processed for enzyme terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using an In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cytocentrifuge preparations and cryostat sections, fixed in 4% paraformaldehyde, were treated with Triton X-100 (0.1%) at 4°C for two minutes. Then, the preparations were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT. Finally, the preparations were washed with PBS, mounted in a glycerin-PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazobicyclo-2.2.2-octane (DABCO) (Janssen Chimica) and apoptotic cells were detected and enumerated by fluorescence microscopy (Leica DM RBE, Wild Leitz).

Double-labeling experiments. Double-labeling experiments were conducted to determine the relation between PRRSV replication and apoptosis. Briefly, cytocentrifuge preparations and cryostat sections, fixed in 4% paraformaldehyde, were treated with Triton X-100 (0.1%) at 4°C for two minutes. The preparations were first incubated with a pool of monoclonal antibodies (MAb) against the PRRSV nucleocapsid protein (dilution 1/100 of WBE1 and WBE4-6) (Drew et al., 1995). Subsequently, the preparations were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT and then incubated with goat anti-mouse TexasRed (dilution 1/100) (Amersham).

Double-labeling experiments were conducted to determine the percentages of apoptotic cells in the subpopulations of $41D3^+$ cells (monocytes/macrophages), $CD2^+$ cells (T lymphocytes and natural killer cells), $CD4^+$ cells (T helper cells), $CD8^+$ cells (cytotoxic T cells and natural killer cells), and IgM^+ cells (B lymphocytes). The percentages in the subpopulation of monocytes/macrophages were determined using MAb 41D3 (Duan et al., 1998). Briefly, cytocentrifuge preparations of BAL cells and cryostat sections of lung tissue, fixed in 4% paraformaldehyde, were treated with Triton X-100 (0.1%) at 4°C for two minutes. The preparations were first incubated with MAb 41D3 (dilution 1/100). Subsequently, the preparations were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT and then incubated with goat anti-mouse TexasRed (dilution 1/100) (Amersham). The percentages of apoptotic cells in the subpopulations of $CD2^+$, $CD4^+$, and $CD8^+$ cells were determined using MAbs MSA4 (Pescovitz et al., 1994a), 74-12-4 (Pescovitz et al., 1994b), and 76-2-11 (Zuckermann et al., 1998), respectively. The percentages in the subpopulation of B lymphocytes were determined using MAb 76-7-4 (Denham et al., 1994). Briefly, cytocentrifuge preparations of BAL cells were first incubated with the respective MAbs and subsequently with goat anti-mouse TexasRed (dilution 1/100) (Amersham). Then, the preparations were treated with Triton X-100 (0.1%) at 4°C for two minutes. Subsequently, the preparations were subjected to an enzymatic incorporation of digoxygenin-labeled nucleotide with TdT.

Cytokine bioassays and ELISA. IL-1 was determined in a proliferation assay using D10(N4)M cells (Hopkins & Humphreys, 1989, 1990). One unit of IL-1 activity was defined as the reciprocal of the dilution producing 50% proliferation of D10(N4)M cells.

To assign bioactivity to IL-1, monoclonal rat anti-mouse IL-1 receptor type 1 antibodies (R&D Systems) were included in the assay.

TNF-α was determined in a cytotoxicity assay in PK(15) subclone 15 cells (Bertoni et al., 1993). One unit of TNF-α activity was defined as the reciprocal of the dilution producing 50% cytotoxicity. Cytotoxic activity was confirmed to be induced by TNF-α by neutralization of samples with rabbit anti-human TNF-α (Innogenetics).

IL-10 was determined in a commercial ELISA for porcine IL-10 (Biosource International) according to the manufacturer's instructions.

***In vitro* experiments.** Porcine peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Paque^{TM} Plus (Amersham Biosciences) density sedimentation. PBMCs were seeded in 24-well cell culture plates (Nunc) at a concentration of 10^7 cells per well. Monocytes were isolated from PBMCs by adherence on culture flask for 2 hours at 37°C, 5% CO_2. Non-adherent cells were removed by washing and the adherent cells were cultured at 37°C overnight. After an additional washing, the final adherent population was used as the source of blood monocytes. Porcine alveolar macrophages were obtained by means of a broncho-alveolar lavage and seeded in 24-well cell culture plates (Nunc) at a concentration of 10^6 cells per well. One day after seeding, blood monocytes and alveolar macrophages were treated with recombinant porcine IL-1β (gift from A. Billiau, Leuven, Belgium) at a concentration of 200 U/ml (75 x10^3 ng/ml) or with recombinant porcine IL-10 (Biosource International) at concentrations of 1, 10, or 100 ng/ml. Functional activity of both recombinant porcine IL-1β and recombinant porcine IL-10 had been proven in a proliferation assay using D10(N4)M cells (Hopkins & Humphreys, 1989, 1990) and the mouse mast cell line D36 (Schlaak et al., 1994), respectively. Untreated cells were included as controls. Morphological analysis of the cells was performed daily with a contrast-phase Olympus microscope. Cells were harvested at 96 hours after treatment. Cytocentrifuge preparations were made, fixed in 4% paraformaldehyde for 10 minutes at room temperature and processed for enzyme terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using an In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim) according to the manufacturer's instructions. Apoptotic cells were detected and enumerated by fluorescence microscopy (Leica DM RBE, Wild Leitz)

Results

Quantification of PRRSV-infected and apoptotic cells in lung tissue. The quantification of infected and apoptotic cells in lung tissue throughout a PRRSV infection is presented in Figure 1. PRRSV-infected cells were observed from 3 until 25 days PI with a maximal number of 61 cells/100 mm^2 at 7 days PI. Viral antigen-positive foci, defined as areas in lung tissue containing groups of three or more PRRSV-infected cells and cellular debris (Labarque et al., 2000), were observed between 3 and 14 days PI with a maximal number of 37 foci/100 mm^2 at 9 days PI (not shown).

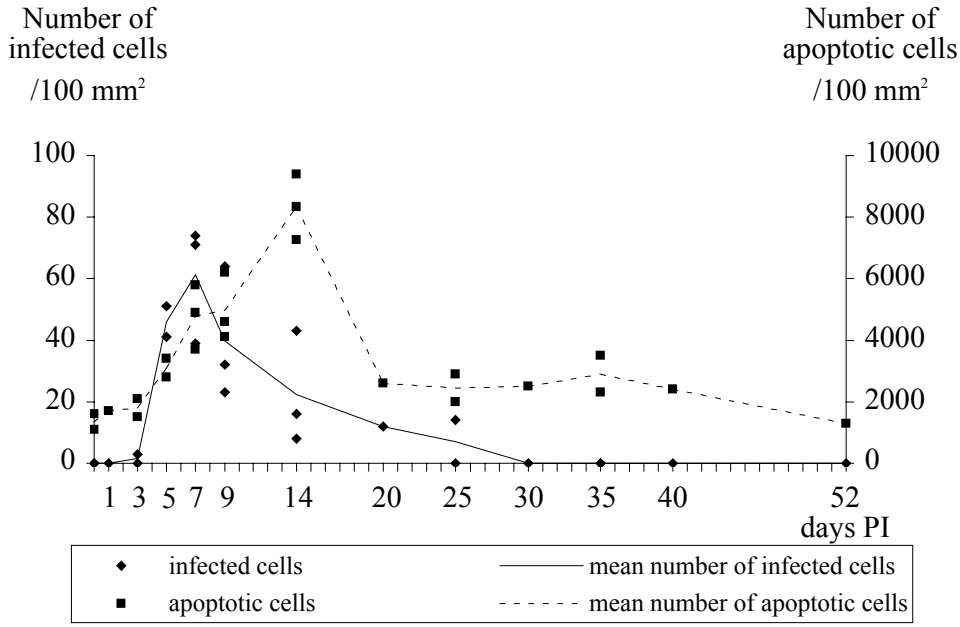

Fig. 1. Quantification of infected and of apoptotic cells in lung tissue (/100 mm^2) throughout a PRRSV infection.

Apoptotic cells were detected in lung tissue of both non- and PRRSV-inoculated pigs. In non-inoculated control pigs, the mean number was 1350 cells/100 mm^2. In PRRSV-inoculated pigs, mean numbers were similar to those of non-inoculated pigs until 3 days PI. Mean numbers increased from 1800 cells/100 mm^2 at 3 days PI to a level of 4800-4967 cells/100 mm^2 at 7-9 days PI and then peaked at 8330 cells/100 mm^2 at 14 days PI. From 20 days PI, mean numbers returned to those of non-inoculated pigs.

Double-labelings revealed that within the population of infected cells, apoptosis was detected from 5 until 25 days PI with percentages ranging between 9 and 39%. The

majority of apoptotic cells (> 99%) were non-infected. There was a spatial correlation between sites of PRRSV replication and apoptosis. Non-infected apoptotic cells were more abundant in viral antigen-positive foci and they were frequently seen in close proximity to infected cells.

Double-labeling experiments with MAb 41D3 revealed that 76 to 88% of the apoptotic cells were of the monocyte/macrophage lineage.

Quantification of PRRSV-infected and apoptotic cells in the broncho-alveolar spaces. The quantification of infected and apoptotic BAL cells throughout a PRRSV infection is presented in Figure 2. PRRSV-infected cells were first observed at 1 day PI (3 $x10^6$ cells), increased to a maximum of 15 $x10^6$ cells at 9 days PI, decreased to 3 $x10^6$ cells at 14 days PI and remained at levels of 0.5-1 $x10^6$ cells until 40 days PI.

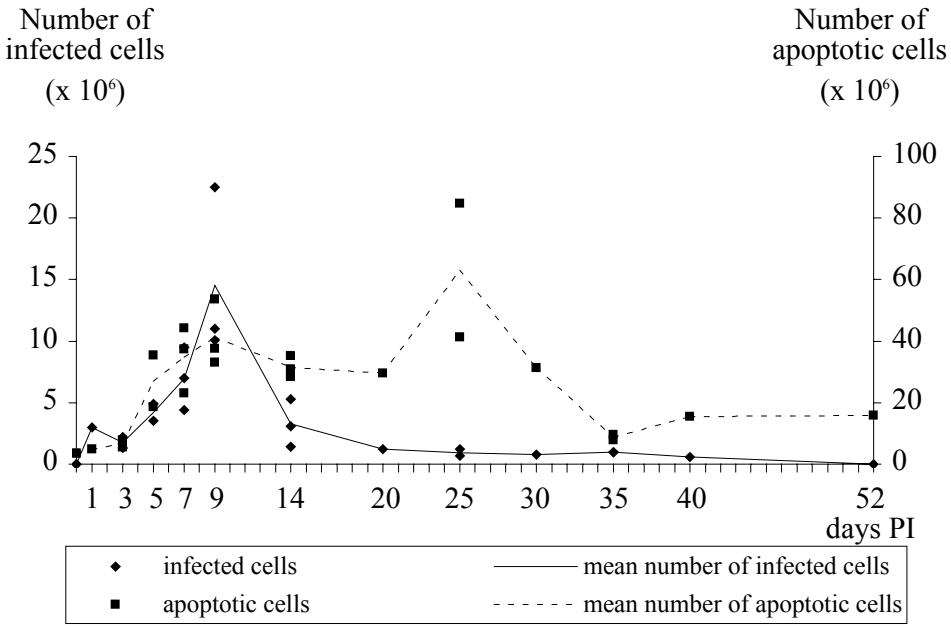

Fig. 2. Quantification of infected and of apoptotic broncho-alveolar lavage (BAL) cells (x 10^6/lung half) throughout a PRRSV infection.

Apoptotic cells were detected in broncho-alveolar spaces of both non- and PRRSV-inoculated pigs. In non-inoculated control pigs, the mean number was 5.3 $x10^6$. In PRRSV-inoculated pigs, mean numbers were similar to those of non-inoculated pigs until 3 days PI. Mean numbers increased from 6.6 $x10^6$ at 3 days PI to a first maximum of 41.4 $x10^6$ at 9 days PI, then remained at the same level until 20 days PI and reached a

second maximum of 63.0 x10^6 at 25 days PI. From 35 days PI onwards, mean numbers of apoptotic cells of PRRSV-inoculated pigs were not different from those of non-inoculated pigs.

Double-labelings revealed that within the population of infected BAL cells, apoptosis was detected until 25 days PI with percentages ranging between 13 and 30%. The majority of apoptotic cells (> 99%) were non-infected.

Mean percentages of apoptotic cells remained constant in the total population of BAL cells and in the subpopulation of monocytes/macrophages (41D3$^+$ cells). In the subpopulation of T lymphocytes and natural killer cells, mean percentages reached a maximum at 7 (CD8$^+$ cells) or 9 days PI (CD2$^+$ and CD4$^+$ cells), dropped thereafter and then remained at constant levels until 52 days PI. In the subpopulation of B lymphocytes (IgM$^+$ cells), apoptotic cells peaked at 5 to 9 days PI and dropped thereafter.

IL-1, TNF-α and IL-10 production in BAL fluids. IL-1, TNF-α and IL-10 levels in BAL fluids throughout a PRRSV infection are presented in Figure 3. A low level of IL-1 (71 U/ml) was found in one out of the seven non-inoculated pigs. The other control pigs had no detectable amounts of IL-1. All PRRSV-inoculated pigs had detectable IL-1 production. The pig euthanized at 1 day PI had an IL-1 titre of 483 U/ml. Mean IL-1 titres increased from 240 U/ml at 3 days PI to a maximum of 1265 U/ml at 9 days PI, decreased to 263 U/ml at 14 days PI and remained at levels of 121-267 U/ml until 52 days PI.

All non-inoculated control pigs were negative for TNF-α. After PRRSV inoculation, TNF-α was only found in the three pigs euthanized at 14 days PI, at low levels (32-109 U/ml).

All non-inoculated control pigs were negative for IL-10. After PRRSV inoculation, IL-10 was found starting from 5 days PI (19 pg/ml). Mean IL-10 levels reached a maximum of 139 pg/ml at 9 days PI, decreased to 14 pg/ml at 14 days PI and remained at levels of 8-17 pg/ml until 25 days PI.

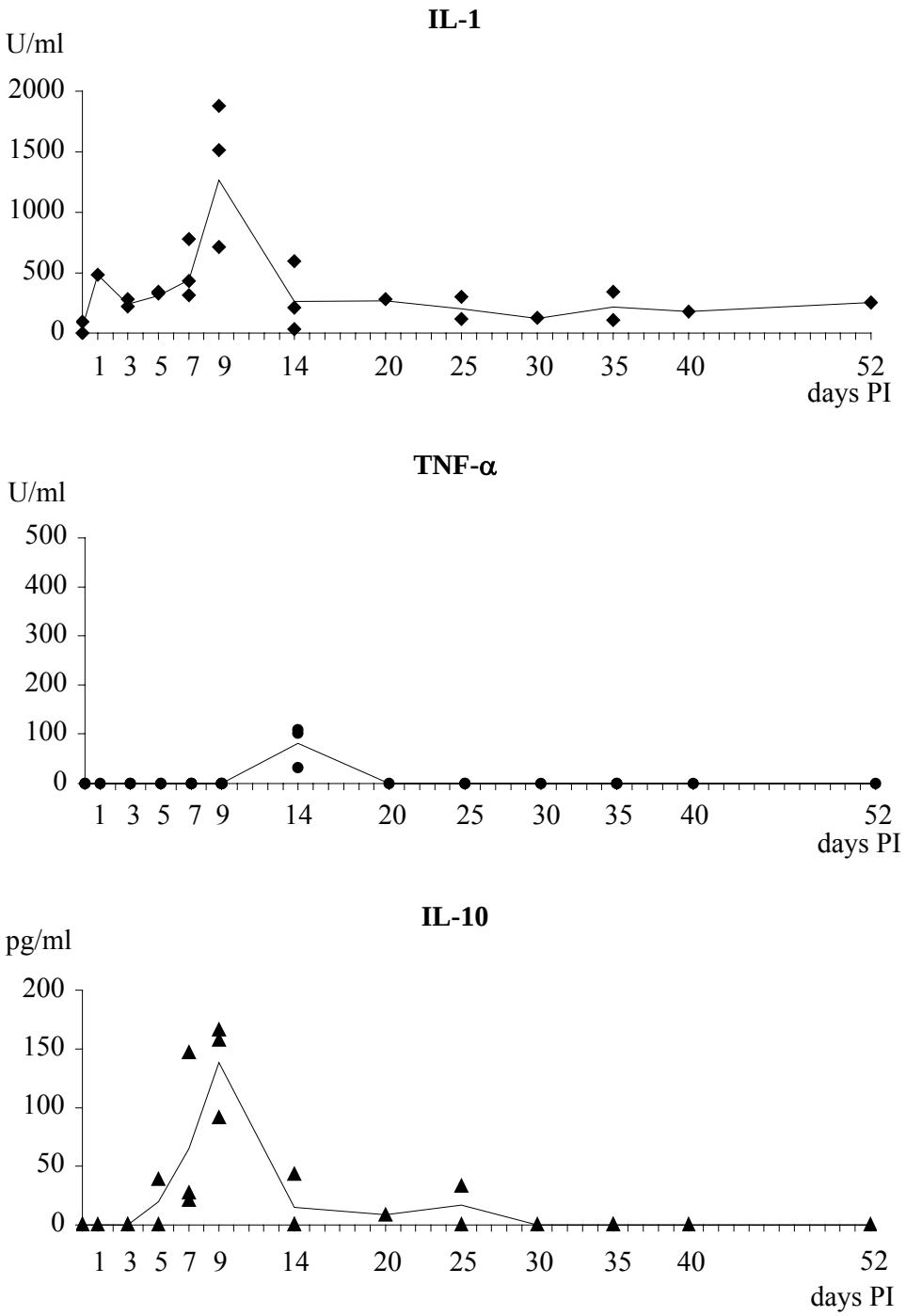

Fig. 3. Interleukin (IL)-1, tumour necrosis factor-alpha (TNF-α) and IL-10 levels in broncho-alveolar lavage (BAL) fluids throughout a PRRSV infection. Dots represent individual cytokine levels; lines represent means.

Quantification of apoptotic cells after *in vitro* treatment with recombinant porcine IL-1β or IL-10. No morphological features of apoptosis, such as cell shrinkage and cytoplasm hypervacuolization, were observed in the monocyte and macrophage cultures throughout the experiment. Four days after treatment with recombinant porcine IL-1β or IL-10, the relative number of apoptotic blood monocytes and alveolar macrophages did not differ from that of untreated cells. Only 2.0% of the untreated blood monocytes and 6.5% of the untreated alveolar macrophages showed apoptosis. After treatment with recombinant porcine IL-1β, percentages of apoptotic blood monocytes and alveolar macrophages were 2.4 and 5.3%, respectively. Similar results were obtained after treatment with the different concentrations of recombinant porcine IL-10. Between 2.5 and 4.0% of blood monocytes and between 5.1 and 6.9% of alveolar macrophages were apoptotic.

Discussion

The present study showed a clear relation between the peak of virus replication and the onset of apoptosis in the lungs of PRRSV-infected pigs. Apoptosis occurred both in virus-infected and in non-infected cells. Most apoptotic cells were non-infected, which is in agreement with previous studies (Sirinarumitr et al., 1998; Sur et al., 1998; Choi & Chae, 2002).

The pattern of apoptosis in the non-infected cells was different in lung tissue compared to broncho-alveolar spaces. In lung tissue, apoptosis peaked at 14 days PI and occurred predominantly in monocytes/macrophages. Earlier studies showed that a PRRSV infection induces a two- to fivefold increase of the number of broncho-alveolar monocytes/macrophages (Labarque et al., 2000). Since apoptosis is a homeostatic process that serves primarily to eliminate redundant cells during normal development and to eradicate defective cells (Metzstein et al., 1998), apoptosis of interstitial monocytes/macrophages throughout a PRRSV infection may be a mechanism of cell population control. However, the process of apoptosis is apparently not sufficient to control completely the massive influx of new monocytes since abundant monocytes/macrophages cross the interstitium towards the broncho-alveolar spaces (Labarque et al., 2000).

The mechanism of apoptosis in non-infected interstitial monocytes/macrophages is not known. It has been suggested that TNF-α may be responsible for the induction of

apoptosis in these cells (Choi & Chae, 2002). However, in the present study, only a very weak TNF-α production was detected at 14 days PI. Our *in vivo* data suggest that IL-1 and IL-10 are better candidates to be involved in the induction of apoptosis in non-infected interstitial monocytes/macrophages. IL-1 and IL-10 levels peaked at 9 days PI and coincided with a significant increase in apoptotic cell numbers. Though, both cytokines probably do not play a role in the induction of apoptosis in non-infected interstitial monocytes/macrophages, since we failed to induce apoptosis in blood monocytes or alveolar macrophages *in vitro* with recombinant porcine IL-1β or IL-10. Using similar *in vitro* experiments, both cytokines were shown to induce apoptosis in human cells. IL-1β was shown to induce apoptosis in a glioblastoma-derived human cell line when concentrations between 1 and 200 U/ml were used (Castigli et al., 2000). Similarly, human monocytes/macrophages underwent apoptosis following treatment with 10 ng/ml of IL-10 (Wang et al., 2001). Thus, the exact mechanism by which PRRSV triggers apoptosis in non-infected interstitial monocytes/macrophages is still under debate. In addition to cytokines, macrophages may produce a lot of other apoptogenic mediators, such as nitric oxide and reactive oxygen species, and any or all of these mediators may contribute to the induction of apoptosis in non-infected monocytes/macrophages. Besides these apoptogenic mediators, viral glycoprotein GP_5, incorporated in cellular debris, may also be responsible for the induction of apoptosis in non-infected cells. This glycoprotein has clearly been shown to be involved in the induction of apoptosis (Suárez et al., 1996). Perhaps, induction of apoptosis can also be alternatively accomplished by attachment of whole virus particles to the cell without penetration and ensuing viral infection. Further, the phagocytic clearance of cellular debris in viral antigen-positive foci by monocytes/macrophages may also be responsible for the apoptosis of non-infected cells. It has been demonstrated that phagocytosis triggers macrophage release of Fas ligand and induces apoptosis in bystander leukocytes (Brown & Savill, 1999). Since monocytes/macrophages themselves express Fas, cross-linking of Fas with soluble Fas ligand may induce apoptosis in non-infected bystander monocytes/macrophages. Both GP_5 and phagocytosis may explain why the highest number of apoptotic cells was found in the proximity of viral antigen-positive foci.

In the broncho-alveolar spaces, two peaks of apoptosis were detected. The first peak at 9 days PI involved mainly lymphocytes. T helper cells ($CD2^+CD4^+CD8^-$), cytotoxic T cells and natural killer cells ($CD2^+CD4^-CD8^+$), as well as B cells (IgM^+) underwent

apoptosis. The percentage of apoptotic cells in T lymphocytes was the highest at the peak of virus replication in BAL cells. This suggests that the PRRSV infection induces apoptosis in broncho-alveolar T lymphocytes and natural killer cells. This is not surprising since T lymphocytes, activated during the antiviral immune response, are predisposed to undergo apoptosis (Razvi & Welsh, 1995). The second peak of apoptosis at 25 days PI can be explained by the fact that the total BAL cell numbers were the highest at that time point, as demonstrated in a previous study (Labarque et al., 2000).

Apoptosis was detected in virus-infected macrophages during the persistent stage of the PRRSV infection. Previous studies have demonstrated that the appearance of a PRRSV-specific immunity causes a significant decrease in the number of viral antigen-positive cells starting at 9 days PI. Nevertheless, a low number of single viral antigen-positive cells persists in the lungs for several weeks (Labarque et al., 2000). Why these single viral antigen-positive cells are able to persist despite the presence of immunity is not known, but may be in part attributable to the apoptotic process in infected cells. It has been suggested that apoptosis of virus-infected cells may represent a very efficient mechanism for viruses to escape from humoral immunity. Progeny virus which is present in apoptotic bodies can be taken up by neighbouring macrophages while protected from antibodies. This type of immune-evasion has already been described for human immunodeficiency virus type 1 (Spetz et al., 1999), herpes simplex virus type 2 (Asano et al., 1999) and Epstein-Barr virus (Holmgren et al., 1999).

Based on the results of the present study, apoptosis may play a significant role in the pathogenesis of a PRRSV infection in the lungs of pigs. Taken together, apoptosis seems to have a homeostatic effect on the number of monocytes/macrophages during the massive influx of new monocytes in the lungs of PRRSV-infected pigs and may explain why a low number of single viral antigen-positive cells persists in the lungs for several weeks despite the presence of immunity.

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4.

EFFECT OF LIPOPOLYSACCHARIDE ON THE LUNGS OF PIGS INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

4.1.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION PREDISPOSES PIGS FOR RESPIRATORY SIGNS UPON EXPOSURE TO LIPOPOLYSACCHARIDE

Veterinary Microbiology (2002) 88, 1-12

Labarque, G., Van Reeth, K., Van Gucht, S., Nauwynck, H. and Pensaert, M.

Summary

This study examined whether an infection with porcine reproductive and respiratory syndrome virus (PRRSV) potentiates respiratory signs upon exposure to lipopolysaccharide (LPS). Five-week-old conventional pigs were inoculated intratracheally with the Lelystad strain of PRRSV and received 5 days later one or two intratracheal LPS administrations. The necessary controls were included. After LPS administration, pigs were intensively monitored for clinical signs. Additionally, some pigs were euthanized after a second LPS administration for broncho-alveolar cell analysis and virological examinations of the lungs. Broncho-alveolar lavage (BAL) cells were counted and differentiated. Lung suspensions and BAL fluids were titrated for PRRSV. Exposure of pigs to PRRSV only resulted in a fever for time periods ranging from 1 to 5 days and slight respiratory signs. Exposure of pigs to LPS only resulted in general signs, characterized by fever and depression, but respiratory signs were slight or absent. PRRSV-LPS exposed pigs, on the other hand, developed severe respiratory signs upon LPS exposure, characterized by tachypnoea, abdominal breathing and dyspnoea. Besides respiratory signs, these pigs also showed enhanced general signs, such as fever and depression. Lung neutrophil infiltration was similar in non-infected and PRRSV-infected pigs upon LPS exposure. PRRSV quantities were similar in lungs and BAL fluids of pigs infected with PRRSV only and PRRSV-LPS exposed pigs. These data show a clear synergy between PRRSV and LPS in the induction of respiratory signs in conventional pigs. The synergy was observed in 87% of the pigs. Thus, it can be considered as reproducible and may be used to test the efficacy of preventive and therapeutic measures.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), an *arterivirus*, causes infections in pigs worldwide. The virus replicates highly in the respiratory tract and shows a distinct tropism for broncho-alveolar macrophages (Duan et al., 1997). However, a single PRRSV infection, particularly under experimental circumstances and with European isolates, fails to induce overt respiratory disease (Done & Paton, 1995). Also under field circumstances, most pigs become infected with PRRSV at growing age without respiratory disease. Still, the frequency and severity of respiratory disease have increased since the enzootic occurrence of PRRSV (Done & Paton, 1995). This has stimulated research into the combined effects of PRRSV and other infectious agents. Consequently, experimentel dual infections have been performed with PRRSV followed by various bacteria such as *Haemophilus parasuis*, *Pasteurella multocida*, *Salmonella* Choleraesuis, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, and *Bordetella bronchiseptica* (Galina et al., 1994; Cooper et al., 1995; Pol et al., 1997; Brockmeier et al., 2000). We ourselves have performed dual infections with PRRSV followed by enzootic viruses, notably porcine respiratory coronavirus (PRCV) or swine influenza virus (SIV) (Van Reeth et al., 1996). The clinical effects of these combinations were extremely severe in some cases, but almost completely subclinical in other. Most important, none of the dual infections mentioned provides a reliable model to study pathogenetic features or to test control measures. We hypothesized therefore that the clinical outcomes of dual inoculations with two infectious agents are influenced by factors that are very difficult to control, such as the stage of replication and the viral or bacterial load.

Lipopolysaccharides (LPS) or endotoxins, a major constituent of the cell wall of Gram-negative bacteria, are released in high concentrations in the lungs upon infection with Gram-negative bacteria (Pugin et al., 1992; Lamp et al., 1996; Kadurugamuwa & Beveridge, 1997) and these endotoxins are present in varying concentrations in dust in swine buildings (Rylander, 1994; Zejda et al., 1994; Zhiping et al., 1996). The release of LPS by Gram-negative bacteria, such as *Actinobacillus pleuropneumoniae* and *Bordetella bronchiseptica* may explain the more severe disease in the experimental dual infections with PRRSV and these bacteria (Pol et al., 1997; Brockmeier et al., 2000). Van Reeth et al. (2000) recently demonstrated that dual inoculations with PRCV followed by LPS seriously aggravate respiratory signs in gnotobiotic pigs, while the respective single

inoculations were subclinical. Therefore, we wanted to examine if exposure of PRRSV-infected pigs to LPS similarly enhances respiratory signs. PRRSV may lend itself excellently as a predisposing agent for synergy with LPS, because all pigs become infected at ages varying from 4 weeks to fattening age (Albina et al., 1994; Houben et al., 1995). Also, PRRSV persists in the lungs for 40 (Labarque et al., 2000) to 49 (Mengeling et al., 1995) days after inoculation. We have examined the clinical course of inoculations with PRRSV followed by LPS, and the effect of the timing and frequency of LPS administrations. Additionally, some preliminary investigations of cellular and virological aspects in the lungs were performed.

Materials and Methods

Virus and LPS. A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV (Wensvoort et al., 1991) was used in this study. The inoculation dose was $10^{6.0}$ $TCID_{50}$/pig.

Escherichia coli LPS (O111:B4) was obtained from Difco Laboratories and used at a dose of 20 μg/kg body weight. This dose was based on data from previous experiments in gnotobiotic pigs, and selected to cause no respiratory signs (Van Reeth et al., 2000).

Pigs and experimental design. Forty-six conventional pigs, originating from twelve PRRSV-negative sows, were used. Pigs were weaned at 4 weeks of age and placed in isolation. They were allowed to acclimatize during 7 days before initiation of the experiments. PRRSV inoculations and LPS administrations occurred intratracheally as described by Van Reeth et al. (2000). Briefly, the pigs were held in vertical position with their neck extended. A needle was inserted through the skin cranial to the sternum and the inoculum was injected. The intratracheal administration was chosen to ensure that all pigs received exactly the same dose in the lungs. Three experiments were performed.

In a first experiment, fifteen pigs were inoculated with PRRSV and received one LPS administration 5 days later. Seven pigs were inoculated with PRRSV only. Eight pigs, not previously inoculated with PRRSV, received one LPS administration. Clinical monitoring was performed daily during 5 consecutive days after PRRSV inoculation and every 2 hours during the first 12 hours after LPS administration.

In a second experiment, eight pigs were inoculated with PRRSV and, 5 days later, they received two LPS administrations with a 3-hour interval. Four pigs, not previously

inoculated with PRRSV, received two LPS administrations with a 3-hour interval. Clinical monitoring was performed daily during 5 consecutive days after PRRSV inoculation and at 1, 3, 5, 7, and 9 hours after the second LPS administration.

In a third experiment, eleven out of the fifteen PRRSV-LPS exposed pigs, described in the first experiment, received a second LPS administration, 24 hours after the first one. These pigs were divided in two subgroups. One subgroup of six pigs was again monitored for clinical signs every 2 hours until 12 hours after the second LPS administration. One subgroup of five pigs was euthanized between 5 and 7 hours after the second LPS administration for study of the broncho-alveolar lavage (BAL) cell population and for virological and bacteriological examinations of the lungs. From the seven PRRSV control pigs, described in the first experiment, four pigs were again monitored for clinical signs every two hours for 12 hours at day 6 after PRRSV inoculation. The remaining three pigs were euthanized at time points corresponding to those of the PRRSV-LPS exposed pigs and served as controls for the broncho-alveolar cell and virological examinations. All eight LPS-exposed pigs, described in the first experiment, received a second LPS administration, 24 hours after the first one. Four pigs were again monitored for clinical signs every 2 hours until 12 hours after the second LPS administration and four pigs were euthanized between 5 and 7 hours after the second LPS administration for broncho-alveolar cell and virological examinations. Four non-inoculated pigs were euthanized for the same purpose.

Clinical monitoring. Pigs were monitored for general signs, notably fever and depression, and for respiratory signs, notably tachypnoea, abdominal breathing and dyspnoea. Scores were given for these five clinical parameters. Body temperatures of ≤39.9°C were scored as 0, temperatures between ≥40.0°C and ≤40.9°C were scored as 1 and temperatures of ≥41.0°C were scored as 2. Respiration rates of ≤45 were scored as 0, rates between ≥46 and ≤59 were scored as 1 and rates of ≥60 were scored as 2. Depression, abdominal breathing and dyspnoea were each scored as 0 (absent) or 1 (present). Scores were added up and a mean of the cumulative general and respiratory scores per group was calculated.

Broncho-alveolar cell examinations. At necropsy, the lungs were removed. The right lung was used for broncho-alveolar cell examination after broncho-alveolar lavage using

the method described by Van Reeth et al. (1998). The BAL fluid was centrifuged (400xg, 10 minutes, 4°C) to separate the cells and the cell-free lavage fluid. Aliquots of the cell-free lavage fluid were stored at –70°C until virus titration on PAMs. BAL cells were counted in a Türk chamber and cytocentrifuge preparations were stained with DiffQuik (Baxter) to determine the percentage of mononuclear cells and neutrophils.

Virological and bacteriological examinations. The left lung was used for virological and bacteriological examinations. Twenty percent suspensions of lung lobes were made in phosphate-buffered saline, clarified by centrifugation and the supernatant was used for PRRSV titration. Virus titration of lung suspensions and of cell-free BAL fluids was performed on PAMs, as described by Labarque et al. (2000).

For bacteriology, samples of lung tissue were plated on bovine blood agar and cultured aerobically. A nurse colony of coagulase-positive *Staphylococcus* species was streaked diagonally on each plate. Plates were inspected for bacterial growth after 48 and 72 hours.

Statistical analysis. Non-parametric tests were used, because of lack of normality in the data. Standard two-sample Mann-Whitney tests were used to compare general and respiratory clinical scores between groups. $P<0.05$ was taken as the level of statistical significance. Statistical analyses were performed using SPSS 6.1.

Results

Clinical signs after PRRSV only. Twenty-six of the total of thirty PRRSV-infected pigs developed fever for time periods ranging from 1 to 5 days. Respiratory signs were absent, except for two pigs, which showed tachypnoea and abdominal breathing.

In experiment 1, six of the seven PRRSV control pigs showed fever until the end of the monitoring period. Respiratory signs were slight in one pig and absent in the other pigs. The mean respiratory score was 1.0 (Table 1).

In experiment 3, all four PRRSV control pigs showed fever until the end of the monitoring period. Respiratory signs, characterized by increased respiration rates, were observed in one of the four pigs. The mean respiratory score was 2.5 (Table 1).

Table 1. Mean general and respiratory scores after the last LPS administration in PRRSV-LPS exposed pigs and their controls.

Exp.	Exposure	No. of pigs	Mean ± S.D. of the cumulative … scores	
			general[1]	respiratory[2]
1	PRRSV-5d-LPS	15	10.0 ± 3.4^{A}	11.8 ± 8.0^{A}
	PRRSV	7	6.0 ± 2.9^{B}	1.0 ± 2.6^{B}
	LPS	8	4.0 ± 1.5^{B}	0.6 ± 1.8^{B}
2	PRRSV-5d-LPS-3h-LPS	8	8.8 ± 3.7^{A}	15.5 ± 6.0^{A}
	LPS-3h-LPS	4	4.3 ± 1.9^{B}	7.0 ± 5.5^{B}
3	PRRSV-5d-LPS-24h-LPS	6	9.8 ± 3.1^{A}	11.0 ± 4.2^{A}
	PRRSV	4	8.5 ± 0.6^{A}	2.5 ± 5.0^{AB}
	LPS-24h-LPS	4	0.5 ± 0.6^{B}	0.0 ± 0.0^{B}

[1]: body temperature (0: ≤39.9°C; 1: ≥40.0°C-≤40.9°C; 2: ≥41.0°C) and depression (0: absent; 1: present)
[2]: respiration rate/minute (0: ≤45; 1: ≥46-≤59; 2: ≥60), abdominal breathing (0: absent; 1: present) and dyspnoea (0: absent; 1: present)
[A,B]: Within each experiment, values with different superscripts in the same column are significantly different by standard two-sample Mann-Whitney test ($P<0.05$)

Clinical signs after one LPS administration. In non-infected pigs, a single LPS administration induced transient general signs (Figure 1). Respiratory signs were slight or absent and the mean respiratory score was only 0.6 (Table 1). In PRRSV-infected pigs, however, LPS induced severe clinical signs with fever in all pigs and respiratory signs in 87% of the pigs (Figure 1). Respiratory signs were characterized by tachypnoea (peak 154 breaths per minute), abdominal breathing and dyspnoea and lasted until the end of the monitoring period. Two out of fifteen pigs did not show respiratory signs after LPS administration. Mean general and respiratory scores were significantly higher in PRRSV-LPS exposed pigs than in singly LPS-exposed pigs (Table 1).

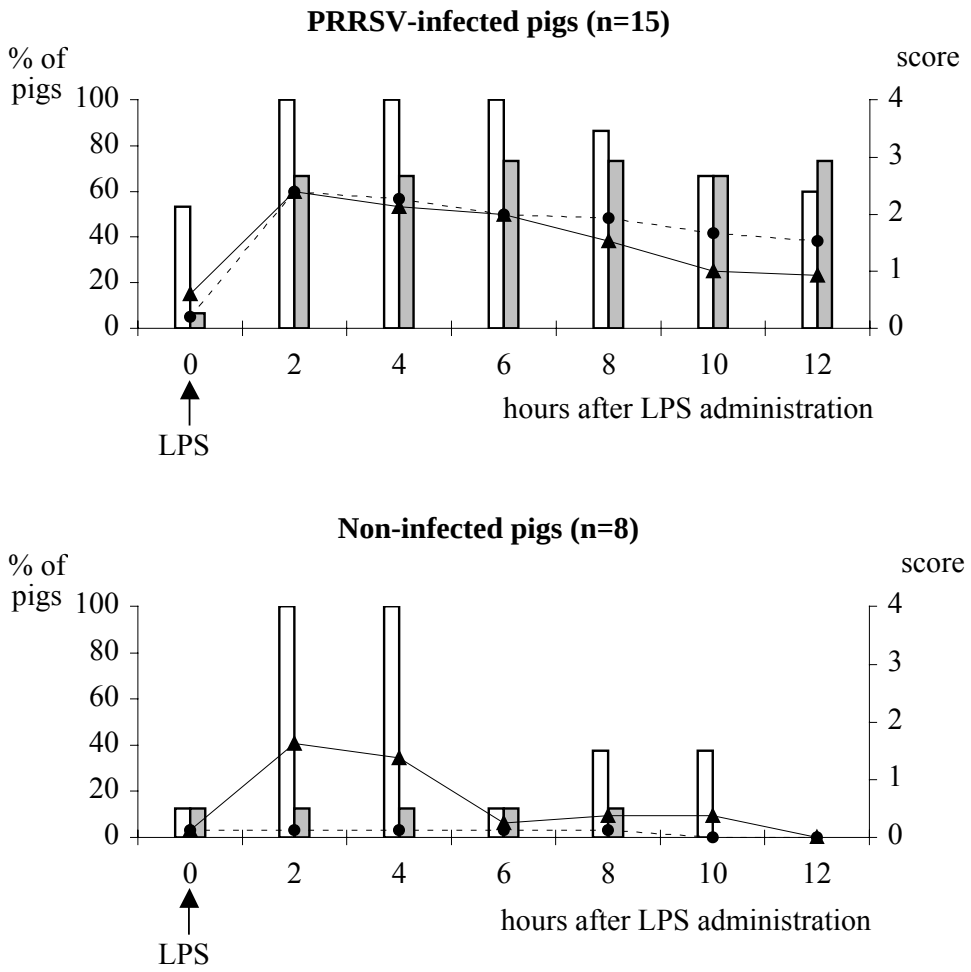
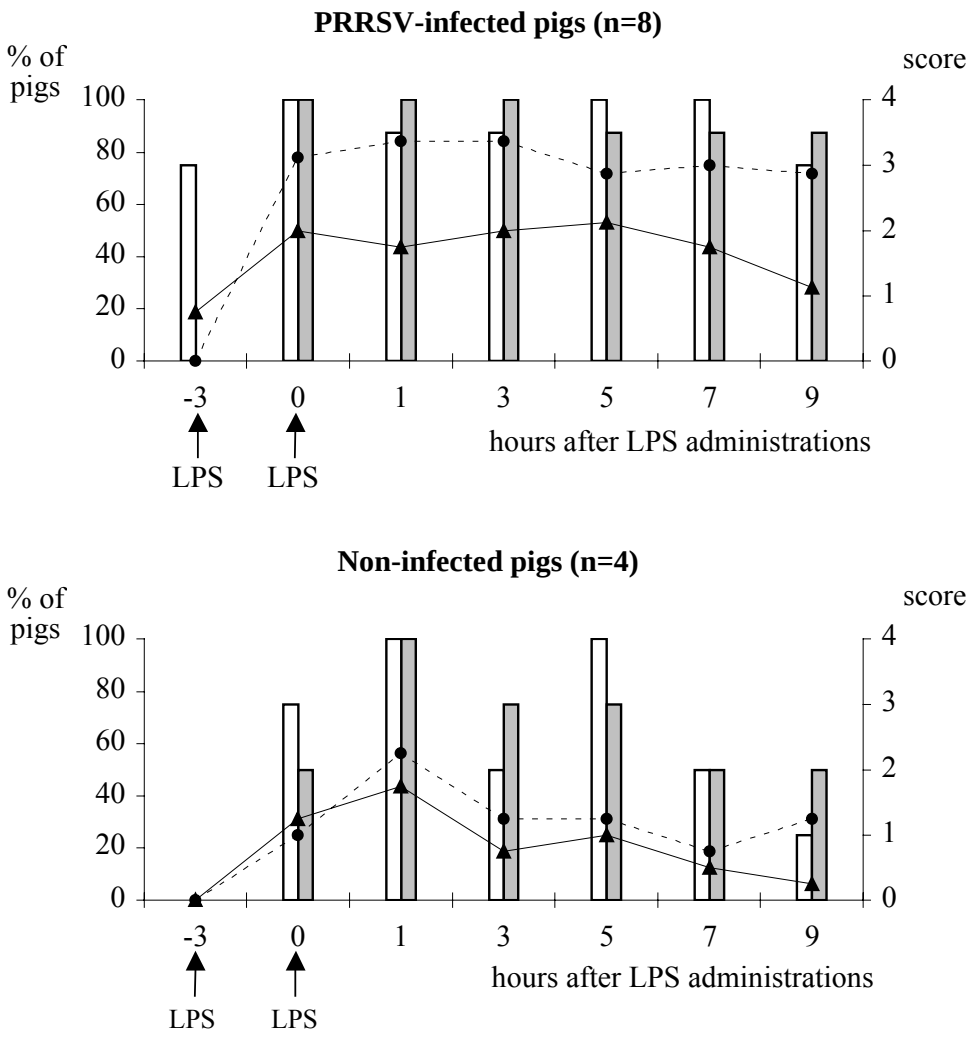


Fig. 1. Evolution of clinical signs after one LPS administration in PRRSV-infected and non-infected pigs.

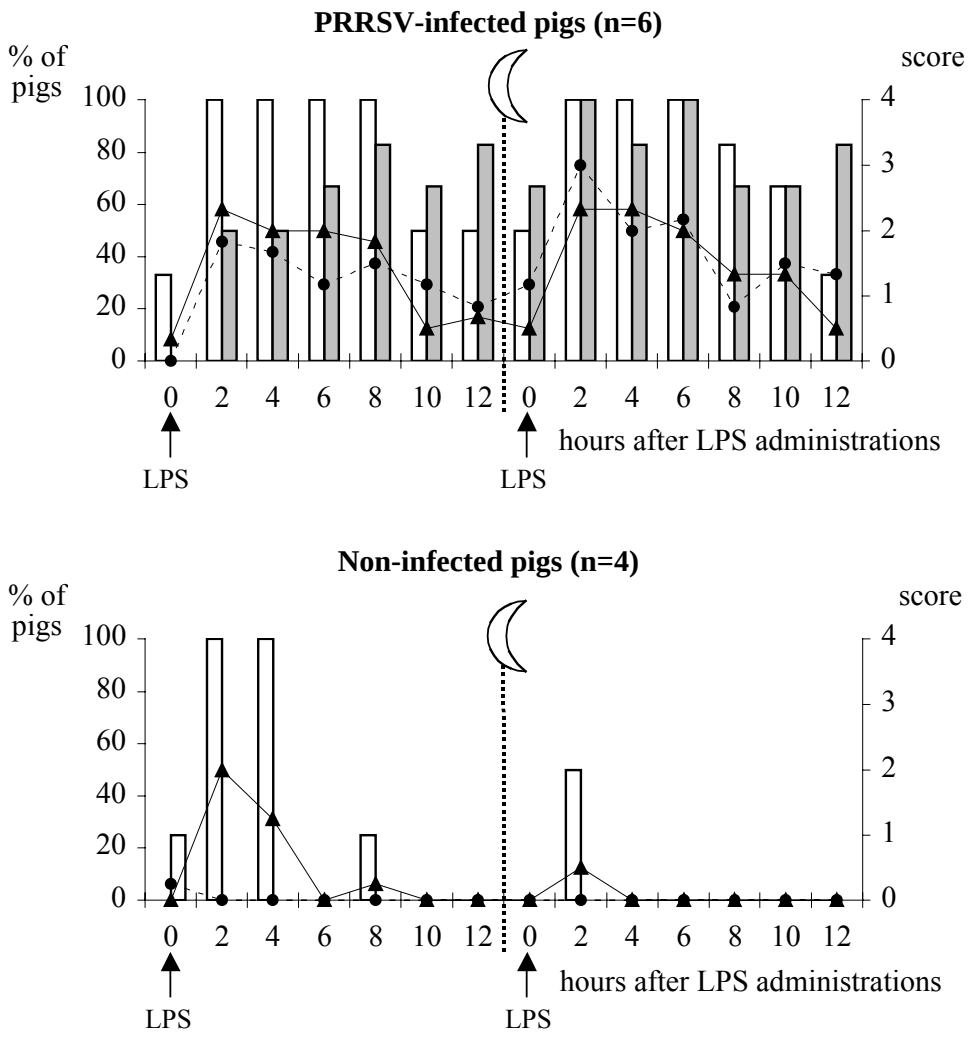
Clinical signs after two LPS administrations with a 3-hour interval. In non-infected pigs, which received two LPS administrations with a 3-hour interval, both general and respiratory signs were observed (Figure 2). Clinical signs were significantly higher in PRRSV-infected pigs, not only with regard to the number of affected pigs but also with regard to the clinical scores. All pigs reacted severely. The mean clinical scores after the second LPS administration are presented in Table 1.



for legend: see Fig. 1.

Fig. 2. Evolution of clinical signs after two LPS administrations with a 3-hour interval in PRRSV-infected and non-infected pigs.

Clinical signs after two LPS administrations with a 24-hour interval. Non-infected pigs had recovered at the time of the second LPS administration, 24 hours after the first one. This second LPS administration again induced general signs within 2 hours, but no respiratory signs (Figure 3). PRRSV-infected pigs had not yet recovered 24 hours after the first LPS administration (Figure 3). The second LPS administration, however, increased the number of pigs with general and respiratory signs and mean clinical scores (Figure 3). Here again, mean general and respiratory scores were significantly higher in PRRSV-LPS exposed pigs than in singly LPS-exposed pigs.



for legend: see Fig. 1.

Fig. 3. Evolution of clinical signs after two LPS administrations with a 24-hour interval in PRRSV-infected and non-infected pigs.

Lung inflammatory findings. Total BAL cell numbers and differentials are shown in Table 2. BAL cell numbers and differentials in PRRSV-LPS exposed pigs were essentially similar to those of pigs, exposed to PRRSV or LPS only. However, there was great individual variation within all three groups.

Table 2. Broncho-alveolar lavage (BAL) cell study of PRRSV-LPS exposed pigs and their controls at 5-7 hours after a second LPS administration.

Exposure	No. of pigs	BAL cells		
		Total (x 10^6) mean (range)	Differentiation (%) monocytes/macrophages mean (range)	neutrophils mean (range)
PRRSV-LPS	5	1874 (600-3360)	35 (20-46)	60 (46-76)
PRRSV only	3	1087 (500-1500)	67 (54-83)	24 (8-36)
LPS only	4	1698 (700-2180)	37 (28-39)	59 (48-67)
none	4	510 (460-560)	93 (88-96)	0.5 (0-1)

Mean PRRSV titres in lungs and BAL fluids are shown in Table 3. Virus titres were similar in lungs and BAL fluids of singly PRRSV-inoculated pigs and PRRSV-LPS exposed pigs. The lungs and BAL fluids of LPS controls and non-inoculated controls were negative for PRRSV.

Bacterial culture of lung tissue yielded negative results for all pigs.

Table 3. Virological study of lungs and broncho-alveolar lavage (BAL) fluids of PRRSV-LPS exposed pigs and their controls at 6 days after PRRSV inoculation.

Exposure	No. of pigs	Mean PRRSV titres (range) in …	
		lung tissue (log_{10} $TCID_{50}$/g)	BAL fluids (log_{10} $TCID_{50}$/ml)
PRRSV-LPS	5	7.1 (6.3–7.9)	6.5 (5.8–7.8)
PRRSV only	3	6.5 (5.8-7.2)	6.0 (4.8-6.6)
LPS only	4	negative	negative
none	4	negative	negative

Discussion

It has become generally accepted that PRRSV plays an important role in respiratory disease problems in the field, particularly in multi-factorial respiratory disease. However, it has been most difficult to reproduce respiratory signs in experimental infections studies

with PRRSV and a second infectious agent. The present PRRSV-LPS combination induces clear respiratory signs in 87% of the pigs. Unlike in our previous studies with PRRSV-SIV and PRRSV-PRCV combinations (Van Reeth et al., 1996), mean clinical scores were higher than those of control pigs in each experiment. Only two out of fifteen PRRSV-infected pigs did not develop respiratory signs upon LPS exposure. It is important to mention, however, that individual variation in disease severity is unavoidable with respiratory pathogens. Such an individual variation has even been reported in experimental infection studies with primary respiratory pathogens such as *Actinobacillus pleuropneumoniae* (Baarsch et al., 2000) or swine influenza virus (Van Reeth et al., 1998).

We used two LPS administrations with the purpose to extend the duration of clinical signs. The clinical effect of a second LPS administration was dependent on the time interval between the two LPS administrations. In non-infected pigs, a second LPS administration at a 24-hour interval caused milder clinical signs than the first one. On the other hand, a second LPS administration within a 3-hour interval seriously aggravated and prolonged clinical signs. These observations suggest that two LPS administrations within a short time interval lead to an accumulation of LPS in the lungs. Indeed, it has been demonstrated that the clinico-pathological manifestations of LPS are strictly dose-dependent. For example, if sufficient amounts of LPS are given to animals and man, cytokine induction, lung inflammation and decreased lung function are observed. Slightly smaller LPS doses, on the other hand, will cause only a mild lung inflammation (Michel et al., 1997). In PRRSV-infected pigs, the clinical effect of a second LPS administration was difficult to assess since pigs had not yet recovered at the moment of the second LPS administration, 3 or 24 hours after the first one. There is little information on the effect of repeated LPS administrations to farm animals in the literature. It appears logical, however, that the mediators or mechanisms responsible for the clinical effects of LPS may become exhausted if high LPS doses are administered frequently.

Respiratory signs following PRRSV-LPS exposure could not be explained by the degree of virus replication or inflammatory changes in the lungs. Indeed, virus titres were similar in PRRSV-LPS or singly PRRSV-inoculated pigs. Total BAL cell numbers and neutrophil infiltration were similar in PRRSV-LPS or singly LPS-exposed pigs. Also, the two PRRSV-LPS exposed pigs, which remained healthy, had similar BAL cell profiles as their clinically affected group mates. This suggests that inflammatory changes

in the lungs have little or no effect on the synergy between PRRSV and LPS. We hypothesize, therefore, that functional lung changes, such as bronchial hyper-responsiveness, are more important in the pathogenesis of PRRSV-LPS induced disease than structural changes. Similar findings were made in a previous experimental infection study with PRCV followed by LPS (Van Reeth et al., 2000). In this study, disease development was tightly correlated with lung production of pro-inflammatory cytokines, among which tumour necrosis factor-alpha (TNF-α). Interestingly, TNF-α has been shown to cause bronchial hyper-responsiveness in laboratory animal models and humans (Kips et al., 1992; Thomas et al., 1995).

Under field circumstances, most pigs become infected with PRRSV at growing age and they are continuously exposed to airborne endotoxins. Also, during Gram-negative infections of the lungs, excessive amounts of endotoxins are released locally. The present PRRSV-LPS infection model, therefore, is relevant for the study of PRRSV-induced respiratory problems in the field. The synergy was observed in 87% of the pigs. Thus, it can be considered as reproducible and may be used to test the efficacy of preventive and therapeutic measures.

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4.2.

REPLICATION OF ATTENUATED PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS STRAINS IN LUNGS AND SENSITIZATION FOR RESPIRATORY SIGNS UPON EXPOSURE TO LIPOPOLYSACCHARIDE

Summary

Previous studies have shown a clear clinical synergy between the virulent Lelystad strain of porcine reproductive and respiratory syndrome virus (PRRSV) and lipopolysaccharide (LPS). The present study examines whether this synergy also exists with attenuated PRRSV strains. Two experiments were performed. In each experiment, five-week-old conventional pigs were inoculated intratracheally with a commercial modified live PRRSV vaccine, based on either an attenuated American strain or an attenuated European strain. The inoculation doses of both attenuated virus strains were $10^{4.5}$ and $10^{6.0}$ $TCID_{50}$ per pig in experiments 1 and 2, respectively. Pigs inoculated intratracheally with $10^{6.0}$ $TCID_{50}$ of Lelystad virus (only in experiment 2) and non-inoculated pigs were included as controls. At 5 days after virus inoculation, pigs were exposed to *Escherichia coli* LPS (20 µg/kg body weight) by intratracheal route. Clinical signs were recorded every 2 hours from 0 until 12 hours after LPS administration and evaluated by means of a scoring system. Additionally, pigs of the second experiment were euthanized at 12 hours after LPS administration for virological examinations of lung tissue and broncho-alveolar lavage (BAL) fluids (virus titration and quantification of infected cells). Exposure of non-inoculated pigs to LPS resulted in slight (exp. 2; score=1.0) to moderate (exp. 1; score=4.8) respiratory signs. Lelystad virus-inoculated pigs, on the other hand, experienced severe respiratory signs upon LPS exposure (exp. 2; score=14.6), characterized by tachypnoea, abdominal breathing and dyspnoea. Respiratory signs of vaccine virus-LPS exposed pigs were clearly lower than those of Lelystad virus-LPS exposed pigs. Mean scores of pigs inoculated with the attenuated American strain were 6.0 and 8.4 in experiments 1 and 2, respectively. Mean scores of pigs inoculated with the attenuated European strain were 3.0 and 2.6 in experiments 1 and 2, respectively. Mean virus titres and numbers of infected cells in lung tissue and BAL fluids were similar in both vaccine virus-inoculated groups, but clearly lower than those of Lelystad virus-inoculated pigs.

In summary, the present study shows that there exists a clinical synergy between attenuated PRRSV strains and LPS, but the severity is less pronounced than with the virulent Lelystad strain.

Introduction

It has become generally accepted that porcine reproductive and respiratory syndrome virus (PRRSV) plays an important role in respiratory disease problems in the field, particularly in multi-factorial respiratory disease (Done & Paton, 1995). However, it has been most difficult to reproduce respiratory signs in experimental infections with PRRSV and a second infectious agent. Recent research conducted in our laboratory has demonstrated that an infection with the virulent Lelystad strain of PRRSV predisposes pigs for respiratory signs when they are, 5 days later, exposed to lipopolysaccharide (LPS) (Labarque et al., 2002). LPS, a major constituent of the cell wall of Gram-negative bacteria, are released in high concentrations in the lungs upon infection and colonization with Gram-negative bacteria (Pugin et al., 1992; Lamp et al., 1996; Kadurugamuwa & Beveridge, 1997) and are present in varying concentrations in dust in swine buildings (Rylander, 1994; Zejda et al., 1994; Zhiping et al., 1996). Clinical signs upon PRRSV-LPS exposure consisted of fever, depression, tachypnoea, abdominal breathing and dyspnoea whereas control pigs which had been exposed to PRRSV or LPS only, experienced a transient fever with mild or no respiratory signs. The synergy was observed in 87% of the pigs.

Similar to wild-type strains, attenuated PRRSV strains are able to replicate in the lungs of pigs (Thacker et al., 2000). While safety tests with vaccine viruses have yielded satisfactory results under experimental circumstances, it is not excluded that, as with the field virus, replication in the lungs may be predisposing for the appearance of multi-factorial respiratory disease problems under field circumstances. Therefore, safety tests with the vaccine virus alone may not be sufficient and the PRRSV-LPS combination may be required. The purpose of the present study was to examine whether there exists a clinical synergy between attenuated PRRSV strains and LPS. Two commercial modified live PRRSV vaccines, one based on an American virus strain and one based on a European virus strain, were used.

Materials and Methods

Vaccines, virus and LPS. Two commercial modified live PRRSV vaccines were used. One PRRSV vaccine was based on an American virus strain (designated attAm) and the other was based on a European virus strain (designated attEur).

A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV (Wensvoort et al., 1991) was used as virulent virus.

Escherichia coli LPS (O111:B4) was obtained from Difco Laboratories and used at a dose of 20 μg/kg body weight. This dose was based on data from previous studies, and selected to cause mild or no respiratory signs (Van Reeth et al., 2000; Labarque et al., 2002).

Pigs and experimental design. Thirty-five conventional pigs, originating from seven PRRSV-seronegative sows, were used at 5 weeks of age. Virus inoculations and LPS administrations occurred intratracheally. The intratracheal administration was chosen to ensure that all pigs received exactly the same dose in the lungs. Two experiments were performed.

In a first experiment, ten pigs were inoculated with $10^{4.5}$ $TCID_{50}$ of the attenuated American (n=5) or the attenuated European strain (n=5) and were exposed to LPS 5 days later. Five pigs, not previously inoculated with vaccine virus, received one LPS administration and were included as LPS control pigs. Clinical monitoring was performed every 2 hours from 0 until 12 hours after LPS administration.

In a second experiment, ten pigs were inoculated with a higher titre, namely $10^{6.0}$ $TCID_{50}$ of the attenuated American (n=5) or the attenuated European strain (n=5) and were exposed to LPS 5 days later. Five pigs, inoculated with $10^{6.0}$ $TCID_{50}$ of the virulent Lelystad strain and exposed to LPS 5 days later, were included as virulent virus-LPS control pigs. Five pigs, not previously inoculated with vaccine or virulent virus, received one LPS administration and were included as LPS control pigs. Clinical monitoring was performed every 2 hours from 0 until 12 hours after LPS administration and at 12 hours after LPS, all pigs were euthanized for virological examinations. At necropsy, blood and lungs were collected. The right lung was lavaged using the method described by Van Reeth et al. (1998). The broncho-alveolar lavage (BAL) fluid was centrifuged (400xg, 10 minutes, 4°C) to separate the cells and the cell-free lavage fluid. Fractions of the cell-free lavage fluid were stored at –70°C until virus titration. Cell pellets were resuspended in phosphate-buffered saline (PBS) and cytocentrifuge preparations were made by

centrifuging at 140xg for 5 minutes. Samples from the left lung lobes were collected for virological examinations (virus titration and quantification of PRRSV-infected cells).

Clinical monitoring. Pigs were monitored for general signs, notably fever and depression, and for respiratory signs, notably tachypnoea, abdominal breathing and dyspnoea. Scores were given for these five clinical parameters according to a previously described system (Labarque et al., 2002). Briefly, body temperatures of ≤39.9°C were scored as 0, temperatures between ≥40.0°C and ≤40.9°C as 1 and temperatures of ≥41.0°C as 2. Depression was scored as 0 (absent) or 1 (present). Respiration rates of ≤45 were scored as 0, rates between ≥46 and ≤59 as 1 and rates of ≥60 as 2. Abdominal breathing and dyspnoea were each scored as 0 (absent) or 1 (present). Scores were added up and a mean of the cumulative general and respiratory scores per group was calculated.

Virological examinations. Twenty percent suspensions of lung tissue were made in PBS and clarified by centrifugation. Virus titrations of supernatants of the lung suspensions, of cell-free BAL fluids and of serum samples were performed on three-day cultivated MARC-145 cells for both attenuated strains (Bøtner et al., 1999; Stadejek et al., 1999) and on one-day cultivated PAMs (Labarque et al., 2000) for the Lelystad strain.

For the detection of PRRSV-infected cells in BAL cells and lung tissue, a streptavidin-biotin immunofluorescence technique was used. In lung tissue, a distinction was made between single infected cells and viral antigen-positive foci. Viral antigen-positive foci are defined as areas in lung tissue consisting of groups of three or more PRRSV-infected cells and cellular debris (Labarque et al., 2000). Three lung sections per pig were examined. Cytocentrifuge preparations and lung sections were incubated first with monoclonal antibodies (MAbs) against PRRSV, subsequently with biotinylated sheep anti-mouse antibodies (Amersham) (dilution 1/200), and then with streptavidin-fluorescein isothiocyanate (FITC) (Amersham) (dilution 1/200). The MAbs used were WBE1 and WBE4-6 (Drew et al., 1995) for the Lelystad virus-inoculated pigs, MR40 (Nelson et al., 1996) for the pigs inoculated with the attenuated American strain and P3/27 (Wieczorek-Krohmer et al., 1996) for the pigs inoculated with the attenuated European strain.

Statistical analysis. Standard two-sample Mann-Whitney tests were used to compare clinical scores between groups. Differences in numbers of infected cells were analyzed

using an analysis of variance (ANOVA). Least significant differences (LSD) were used to compare the groups. $P<0.05$ was taken as the level of statistical significance. Statistical analyses were performed using SPSS 6.1.

Results

Clinical signs after LPS administration. The means of the cumulative general and respiratory scores after LPS administration are shown in Table 1.

Table 1. Cumulative clinical scores after LPS administration in vaccine virus-inoculated pigs and their controls.

Experiment	Exposure	No. of pigs	Mean ± S.D. of the cumulative ... scores	
			general[1]	respiratory[2]
1	attAm-5d-LPS	5	9.6 ± 2.2^{B}	6.0 ± 3.2^{A}
	attEur-5d-LPS	5	4.2 ± 1.5^{C}	3.0 ± 2.7^{A}
	LPS only	5	1.6 ± 0.5^{A}	4.8 ± 2.8^{A}
2	attAm-5d-LPS	5	8.8 ± 2.2^{A}	8.4 ± 6.5^{BC}
	attEur-5d-LPS	5	8.4 ± 1.9^{A}	2.6 ± 2.4^{AC}
	Lelystad virus-5d-LPS	5	15.0 ± 1.0^{B}	14.6 ± 7.6^{B}
	LPS only	5	6.4 ± 3.8^{A}	1.0 ± 1.0^{A}

[1]: body temperature (0: ≤39.9°C; 1: ≥40.0°C-≤40.9°C; 2: ≥41.0°C) and depression (0: absent; 1: present)
[2]: respiration rate/minute (0: ≤45; 1: ≥46-≤59; 2: ≥60), abdominal breathing (0: absent; 1: present) and dyspnoea (0: absent; 1: present)
[A,B,C]: Within each experiment, values with different superscripts in the same column are significantly different by standard two-sample Mann-Whitney test ($P<0.05$)

Experiment 1 - All LPS control pigs developed general and respiratory signs upon LPS exposure. General signs were characterized by a mild fever, which lasted until 2 to 4 hours after the LPS administration. Respiratory signs, characterized by tachypnoea and dyspnoea, lasted until 6 hours after the LPS administration. The mean respiratory score was 4.8. In pigs inoculated with the attenuated American strain, LPS induced more pronounced clinical signs. All pigs were affected. General signs were characterized by fever and depression and lasted until the end of the monitoring period. Respiratory signs, characterized by tachypnoea and abdominal breathing, lasted until 8 to 10 hours after the LPS administration. The mean respiratory score was 6.0. In pigs inoculated with the attenuated European strain, LPS induced general signs in all pigs and respiratory signs in

four out of the five pigs. General signs were characterized by fever and depression and lasted until 6 to 10 hours after the LPS administration. Respiratory signs, characterized by tachypnoea, lasted until 10 hours after the LPS administration. The mean respiratory score was 3.0. The differences in respiratory scores between LPS control pigs and vaccine virus-LPS exposed pigs were not significant ($P>0.05$).

Experiment 2 - In LPS control pigs, LPS induced mainly general signs. Respiratory signs, characterized by a mild tachypnoea, were observed in three out of the five pigs. The mean respiratory score was 1.0. In Lelystad virus-inoculated pigs, on the other hand, LPS induced severe general and respiratory signs in all pigs. General signs included fever and severe depression. Respiratory signs were characterized by tachypnoea, abdominal breathing and dyspnoea and lasted until the end of the monitoring period. Mean general and respiratory scores were significantly higher than those of LPS control pigs ($P<0.05$). In pigs inoculated with the attenuated American strain, LPS induced clear general and respiratory signs in all pigs. The mean respiratory score (8.4) was significantly higher than that of LPS control pigs ($P<0.05$) and not significantly different from that of Lelystad virus-LPS exposed pigs ($P>0.05$). In pigs inoculated with the attenuated European strain, LPS induced mainly general signs and only slight respiratory signs. The mean respiratory score (2.6) was not significantly different from that of the LPS control pigs ($P>0.05$) and significantly lower than that of the Lelystad virus-LPS exposed pigs ($P<0.05$).

Virus titration of BAL fluids, lung tissue and sera. The results of virus titrations of BAL fluids, lung tissue and sera are shown in Table 2. All LPS control pigs were negative for PRRSV. PRRSV was isolated from BAL fluids and lung tissue of all Lelystad virus-LPS exposed pigs. Mean PRRSV titres were $10^{6.7}$ $TCID_{50}$ per ml BAL fluid and $10^{7.1}$ $TCID_{50}$ per gram lung tissue. Vaccine virus was detected in BAL fluids of four out of the five attAm-LPS exposed pigs and of three out of the five attEur-LPS exposed pigs. Mean virus titres of the positive animals were $10^{4.7}$ and $10^{3.6}$ $TCID_{50}$ per ml BAL fluid for the attAm- and attEur-inoculated pigs, respectively. In lung tissue, three out of the five attAm-LPS exposed pigs and one out of the five attEur-LPS exposed pigs tested positive for virus. Mean virus titres of the positive animals were $10^{4.0}$ and $10^{2.1}$ $TCID_{50}$ per gram lung tissue for the attAm- and attEur-inoculated pigs, respectively. Viraemia was detected in all Lelystad virus- and vaccine virus-inoculated pigs.

Table 2. Virus titres in broncho-alveolar lavage (BAL) fluids, lung tissue and serum samples of vaccine virus-LPS exposed pigs and their controls.

Exposure	Mean virus titres of virus-positive pigs in …		
	BAL fluids (log_{10} $TCID_{50}$/ml)	lung tissue (log_{10} $TCID_{50}$/gram)	serum samples (log_{10} $TCID_{50}$/ml)
attAm-5d-LPS	4.7 (4/5)[1]	4.0 (3/5)	3.0 (5/5)
attEur-5d-LPS	3.6 (3/5)	2.1 (1/5)	2.5 (5/5)
Lelystad virus-5d-LPS	6.7 (5/5)	7.1 (5/5)	3.8 (5/5)
LPS only	negative	negative	negative

[1]: number of virus-positive pigs/total number of pigs

Quantification of PRRSV-infected cells in BAL cells and lung tissue. The results of immunofluorescence stainings of BAL cells and lung tissue are shown in Table 3.

Table 3. Quantification of infected broncho-alveolar lavage (BAL) cells and of single infected cells and/or viral antigen-positive foci in lung tissue of vaccine virus-LPS exposed pigs and their controls.

Exposure	Mean numbers of …		
	infected BAL cells ($x10^6$)	single infected cells in lung tissue (/100 mm^2)	viral antigen-positive foci in lung tissue[1] (/100 mm^2)
attAm-5d-LPS	4.1 (5/5)[2]	8.0 (5/5)	none
attEur-5d-LPS	3.1 (5/5)	4.7 (5/5)	none
Lelystad virus-5d-LPS	45.5 (5/5)	71.2 (5/5)	41.2 (5/5)
LPS only	none	none	none

[1]: areas in lung tissue, consisting of groups of three or more PRRSV-infected cells and cellular debris
[2]: number of positive pigs/total number of pigs

PRRSV-infected cells were not observed in BAL cells and lung tissue of LPS control pigs. PRRSV-infected cells were observed in BAL cells and lung tissue of all vaccine virus-LPS exposed pigs. Mean numbers of infected BAL cells were 4.1 $x10^6$ in attAm-inoculated pigs and 3.1 $x10^6$ in attEur-inoculated pigs. Mean numbers of single PRRSV-infected cells in lung tissue were 8.0 per 100 mm^2 tissue in attAm-inoculated pigs and 4.7 per 100 mm^2 in attEur-inoculated pigs. Mean numbers of infected BAL cells (45.5 $x10^6$)

and of infected cells in lung tissue (71.2 per 100 mm^2) of Lelystad virus-LPS exposed pigs were significantly higher than those of vaccine virus-LPS exposed pigs ($P<0.05$). Viral antigen-positive foci were only observed in lung tissue of Lelystad virus-LPS exposed pigs.

Discussion

The present study demonstrates that a clinical synergy may occur between attenuated PRRSV strains and LPS. Though, the clinical scores of vaccine virus-LPS exposed pigs were clearly lower than those of virulent Lelystad virus-LPS exposed pigs. The differences between virulent virus- and vaccine virus-inoculated pigs may be attributed to the pattern and degree of virus replication in the lungs. Mean virus titres and mean numbers of infected cells were indeed clearly higher in virulent virus-inoculated pigs compared to vaccine virus-inoculated ones. Furthermore, viral antigen-positive foci, consisting of groups of three or more PRRSV-infected cells and cellular debris, were only observed in the lung tissue of virulent virus-inoculated pigs.

There were small differences in clinical scores between both vaccine virus-LPS exposed groups. The attenuated American strain induced more respiratory signs than the attenuated European strain upon LPS exposure. However, due to large variation in between pigs, these differences were not at all statistically significant. All vaccine virus-inoculated pigs were exposed to LPS 5 days after inoculation with the vaccine viruses. This interval was chosen based on the clear clinical synergy between virulent Lelystad virus and LPS (Labarque et al., 2002). It is uncertain, however, whether this 5-day interval is optimal for comparing vaccine viruses. It is possible that vaccine viruses differ from each other and from virulent viruses in their interactions with and in their degree of virus replication in the respiratory tract. To make correct conclusions about differences in clinical synergy between vaccine viruses, the kinetics of replication of vaccine viruses in the lungs and the time point of maximal virus replication should be determined. An LPS administration at that time point will allow definite conclusions. Preliminary virological investigations demonstrated a higher degree of virus replication in the lungs of vaccine virus-inoculated pigs at 10 and 12 days after virus inoculation (data not shown). Therefore, it may be hypothesized that the interval of 5 days between vaccine virus inoculation and LPS administration in the present study may have been too short to sensitize the lungs. This short interval may also explain why virus could not be isolated

from the lungs of some pigs and why the numbers of infected cells in lungs were very low, especially when compared with the data of the Lelystad virus-inoculated pigs.

The PRRSV-LPS combination may be considered as a tool for the safety testing of modified live PRRSV vaccines. However, to that purpose, our PRRSV-LPS model should be ameliorated. Further research, especially with regard to the kinetics of vaccine virus replication in the lungs, has to show whether other time intervals between PRRSV vaccine virus inoculation and LPS administration may allow to use this combination for safety testing of vaccines.

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5.

RESPIRATORY TRACT PROTECTION UPON CHALLENGE OF PIGS VACCINATED WITH MODIFIED LIVE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS VACCINES

Veterinary Microbiology, submitted

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Summary

In this study, the efficacy of two modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccines was assessed in experimental pigs. The virological protection in the lungs of vaccinated pigs upon challenge was studied. Also, challenged pigs were exposed to lipopolysaccharide (LPS) to evaluate clinical protection. Two experiments were performed. In each experiment, six-week-old conventional pigs were immunized intramuscularly with commercial vaccines based on either an attenuated American or an attenuated European virus strain. Non-immunized and infection-immune pigs were included as controls. Six weeks after immunization, pigs were challenged either intratracheally (experiment A) or intranasally (experiment B) with $10^{6.0}$ $TCID_{50}$ of Lelystad virus, and 3 and 6 days later intratracheally exposed to *Escherichia coli* LPS (20 μg/kg body weight). After LPS administration, pigs were monitored for clinical signs. At 4 and 7 days after challenge, some pigs were euthanized to determine virus quantities in broncho-alveolar lavage (BAL) fluids and in lung tissue. Challenge virus was recovered from three out of eight infection-immune pigs that had been inoculated with the virulent Lelystad strain and challenged 6 weeks later with the same virulent strain. The two vaccines reduced virus isolation rates and mean virus titres to different degrees. Fifteen out of sixteen pigs immunized with the attenuated American strain were positive for challenge virus and their mean virus titres were similar to those of the non-immunized challenge controls. Eleven out of sixteen pigs immunized with the attenuated European strain were positive for challenge virus and their mean virus titres were 2.0-2.5 log_{10} lower than those of the non-immunized challenge controls. The clinical outcome upon LPS exposure in vaccinated pigs varied between experiments. In experiment A, pigs immunized with the attenuated American strain experienced severe general and respiratory signs upon LPS exposure, whereas pigs immunized with the attenuated European strain only showed mild general signs. In experiment B, on the other hand, pigs of both immunized groups developed clear general and respiratory signs.

In conclusion, the present study shows that the virological protection in the lungs of infection-immune and vaccinated pigs upon challenge was incomplete, but was more pronounced in the homologous situation. The variable clinical outcome upon LPS exposure in vaccinated pigs within and between both experiments hampers the use of the combined PRRSV-LPS exposure for testing of the clinical efficacy of modified live PRRSV vaccines.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), an *arterivirus*, is widespread in the pig population. The virus replicates highly in the respiratory tract (Duan et al., 1997) and is claimed to play an important role in multi-factorial respiratory disease problems in the field (Done & Paton, 1995). The high economical impact of these problems has stimulated the development of PRRSV vaccines. Currently, several modified live PRRSV vaccines are commercially available for use in feeder pigs. Since experimental infections with European PRRSV isolates fail to induce overt respiratory signs (Plana-Durán et al., 1992; Ramos et al., 1992; Van Reeth et al., 1996), the efficacy of PRRSV vaccines is usually assessed by determining the degree of reduction in viraemia after challenge with a virulent virus (Christopher-Hennings et al., 1997; Nielsen et al., 1997; van Woensel et al., 1998; Labarque et al., 2000b). At present, no information is available about the degree of reduction of challenge virus replication in the respiratory tract. This information would be useful since replication of wild-type virus in the lungs of vaccinated pigs might predispose for multi-factorial respiratory disease problems.

Since a PRRSV infection does not induce respiratory signs, the clinical efficacy of modified live PRRSV vaccines cannot be assessed. This problem may be solved by a combined challenge of PRRSV-vaccinated pigs with PRRSV and lipopolysaccharide (LPS). We have previously demonstrated that infection with the virulent Lelystad strain of PRRSV sensitizes the lungs of pigs for respiratory signs when they are, 5 days later, exposed to LPS (Labarque et al., 2002). Clinical signs consisted of fever, depression, tachypnoea, abdominal breathing and dyspnoea, whereas control pigs, which had been exposed to PRRSV or LPS only, experienced a transient fever and mild or no respiratory signs. The clinical synergy was observed in 87% of the pigs.

It was the purpose of the present study to assess the virological protection in the lungs of vaccinated pigs and to examine if the challenge virus replication in the lungs of these pigs may still predispose for respiratory signs upon LPS exposure. To this purpose, two commercial modified live PRRSV vaccines, one based on an American virus strain and one based on a European virus strain, were tested using a challenge with the European Lelystad strain followed by LPS.

Materials and Methods

Vaccines, virus and LPS. Two commercial modified live PRRSV vaccines and one virulent PRRSV strain were used for active immunization of the pigs. One PRRSV vaccine was based on an American virus strain and the other was based on a European virus strain. A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV (Wensvoort et al., 1991) was used as virulent virus. The inoculation dose of each vaccine or virulent strain was $10^{6.0}$ $TCID_{50}$ per pig.

The virulent Lelystad strain of PRRSV was used as challenge virus. The inoculation dose was $10^{6.0}$ $TCID_{50}$ per pig.

Escherichia coli LPS (O111:B4) (Difco Laboratories; Sigma) was used at a dose of 20 μg/kg body weight. This dose was based on data from previous studies and selected to cause mild or no respiratory signs (Van Reeth et al., 2000; Labarque et al., 2002).

Pigs and experimental design. A total of fifty-eight pigs, originating from eight PRRSV-seronegative sows, were used. Two experiments were performed. In each experiment, pigs were divided into four groups and housed in isolation units. The designation of the groups is shown in Table 1.

At 6 weeks of age, pigs of groups A1 and B1, and of groups A2 and B2, were immunized intramuscularly with one of the two commercial PRRSV vaccines. Pigs of groups A3 and B3 were immunized intramuscularly with the virulent Lelystad strain and served as infection-immune pigs. Pigs of groups A4 and B4 were not immunized and served as challenge control pigs. Six weeks after immunization, all pigs were challenged with Lelystad virus. The challenge was performed intratracheally in experiment A and intranasally in experiment B. At 3 and 6 days after challenge, pigs of all groups were exposed intratracheally to LPS. After LPS administration, clinical signs were recorded and at 24 hours after LPS administration (i.e. at 4 and 7 days after challenge), some of the pigs were euthanized for virological examinations of broncho-alveolar lavage (BAL) fluids, lung tissue and serum samples.

Table 1. Experimental design.

Exp.	Group	Strain used for intramuscular immunization	Route of challenge with Lelystad virus	No. of pigs examined for ...			
				clinical signs*		virus titres	
				3 days PC	6 days PC	4 days PC	7 days PC
A	A1	attAm	intratracheal	6	3	3	3
	A2	attEur	intratracheal	6	3	3	3
	A3	virEur	intratracheal	4	2	2	2
	A4	none	intratracheal	10	5	5	5
B	B1	attAm	intranasal	10	5	5	5
	B2	attEur	intranasal	10	5	5	5
	B3	virEur	intranasal	4	2	2	2
	B4	none	intranasal	8	4	4	4

attAm: attenuated American strain; attEur: attenuated European strain; virEur: virulent European strain (Lelystad virus)
PC: post challenge
*: clinical signs upon intratracheal LPS exposure (20 μg/kg body weight)

Serological examinations. Blood samples of all pigs were collected at the time of immunization and immediately before challenge. Serum samples were examined for PRRSV-specific antibody titres using immunoperoxidase monolayer assays (IPMAs). MARC-145 cells infected with a European PRRSV isolate (94V360) were used. Serum samples of the pigs immunized with the attenuated American strain were also tested in an IPMA with MARC-145 cells infected with an American PRRSV isolate (US5).

Clinical monitoring. Clinical signs were recorded every two hours from 0 until 12 hours after LPS administration. Pigs were monitored for general signs, notably fever and depression, and for respiratory signs, notably tachypnoea, abdominal breathing and dyspnoea. Scores were given for these five clinical parameters according to a previously described system (Labarque et al., 2002). Briefly, body temperatures of ≤39.9°C were scored as 0, temperatures between ≥40.0°C and ≤40.9°C as 1 and temperatures of ≥41.0°C as 2. Depression was scored as 0 (absent) or 1 (present). Respiration rates of ≤45 were scored as 0, rates between ≥46 and ≤59 as 1 and rates of ≥60 as 2. Abdominal breathing and dyspnoea were each scored as 0 (absent) or 1 (present). Scores were added up and a mean of the cumulative general and respiratory scores per group was calculated.

Virological examinations of BAL fluids, lung tissue and serum samples. Blood samples of all pigs were collected at challenge. At necropsy, blood and lungs were collected. The right lung was lavaged using a previously described method (Van Reeth et

al., 1998). The BAL fluid was centrifuged (400xg, 10 minutes, 4°C) to separate the cells and the cell-free lavage fluid. Twenty percent suspensions of the left lung lobes were made in phosphate-buffered saline, clarified by centrifugation and the supernatant was used for PRRSV titration.

Virus titrations of the cell-free BAL fluids, of the supernatants of the lung suspensions, and of the serum samples were performed on PAMs, according to standard procedures (Labarque et al., 2000a).

Statistical analysis. Standard two-sample Mann-Whitney tests were used to compare clinical scores between groups. Differences in PRRSV titres were analyzed using an analysis of variance (ANOVA). Least significant differences (LSD) were used to compare the groups. Samples which tested negative for virus were given a numeric value of 0.2 $\log_{10}$ $TCID_{50}$ per ml BAL fluid or serum (detection limit 0.3 $\log_{10}$) and 0.9 $\log_{10}$ $TCID_{50}$ per gram lung tissue (detection limit 1.0 $\log_{10}$). $P<0.05$ was taken as the level of statistical significance. Statistical analyses were performed using SPSS 6.1.

Results

Serological response to immunization. All pigs were negative for PRRSV-specific antibodies at the start of the experiments. At challenge, all immunized pigs had developed antibodies against PRRSV. Pigs immunized with the attenuated American strain had mean antibody titres of $2^{5.3}$ (group A1) and $2^{5.7}$ (group B1), when a European serotype virus was used in the IPMA. When an American serotype virus was used in the IPMA, these pigs had mean PRRSV-specific antibody titres of $2^{8.3}$ (group A1) and $2^{10.5}$ (group B1). When a European serotype virus was used in the IPMA, pigs immunized with the attenuated European strain had mean antibody titres of $2^{11.7}$ (group A2) and $2^{11.1}$ (group B2), and pigs immunized with the virulent Lelystad strain had mean antibody titres of $2^{11.8}$ (group A3) and $2^{13.3}$ (group B3). Non-immunized pigs (groups A4 and B4) were seronegative at challenge.

Clinical signs upon LPS exposure. Table 2 shows the mean cumulative clinical scores upon LPS administrations at 3 and 6 days after intratracheal (experiment A) or intranasal (experiment B) Lelystad virus challenge.

Table 2. Cumulative clinical scores after LPS administrations at 3 and 6 days after Lelystad virus challenge.

Exp.	Group	Strain used for intramuscular immunization	Route of challenge with Lelystad virus	Time interval challenge - LPS exposure	No. of pigs	Mean ± S.D. of the cumulative … scores after LPS	
						general[1]	respiratory[2]
A	A1	attAm	intratracheal	3 days	6	14.5 ± 1.6^{A}	10.8 ± 4.3^{A}
	A2	attEur	intratracheal		6	3.7 ± 0.8^{B}	0.0 ± 0.0^{B}
	A3	virEur	intratracheal		4	5.0 ± 1.4^{B}	0.0 ± 0.0^{B}
	A4	none	intratracheal		10	12.9 ± 4.0^{A}	9.8 ± 7.2^{A}
	A1	attAm	intratracheal	6 days	3	13.3 ± 2.3^{A}	12.3 ± 7.1^{A}
	A2	attEur	intratracheal		3	0.7 ± 0.6^{B}	0.3 ± 0.6^{B}
	A3	virEur	intratracheal		2	1.0 ± 1.4^{B}	0.0 ± 0.0^{B}
	A4	none	intratracheal		5	9.8 ± 3.6^{A}	9.5 ± 3.1^{A}
B	B1	attAm	intranasal	3 days	10	7.3 ± 1.6^{B}	4.0 ± 3.3^{B}
	B2	attEur	intranasal		10	6.6 ± 1.5^{B}	8.7 ± 4.9^{A}
	B3	virEur	intranasal		4	4.3 ± 4.9^{B}	3.3 ± 3.3^{B}
	B4	none	intranasal		8	12.1 ± 2.6^{A}	14.8 ± 7.4^{A}
	B1	attAm	intranasal	6 days	5	6.0 ± 2.1^{B}	7.6 ± 5.0^{A}
	B2	attEur	intranasal		5	5.0 ± 4.5^{BC}	6.6 ± 4.5^{A}
	B3	virEur	intranasal		2	0.5 ± 0.7^{BC}	1.0 ± 0.0^{A}
	B4	none	intranasal		4	10.0 ± 0.8^{AC}	13.0 ± 9.1^{A}

attAm: attenuated American strain; attEur: attenuated European strain; virEur: virulent European strain (Lelystad virus)
[1]: body temperature (0: ≤39.9°C; 1: ≥40.0°C-≤40.9°C; 2: ≥41.0°C) and depression (0: absent; 1: present)
[2]: respiration rate/minute (0: ≤45; 1: ≥46-≤59; 2: ≥60), abdominal breathing (0: absent; 1: present) and dyspnoea (0: absent; 1: present)
[A,B,C]: Within each experiment, values with different superscripts in the same column are significantly different by standard two-sample Mann-Whitney test ($P<0.05$)

Experiment A - In non-immunized challenged pigs (group A4), LPS induced severe general and respiratory signs in all pigs. General signs were characterized by fever (peak 42.0°C) and severe depression. Pigs were dull and made no effort to rise when disturbed. Respiratory signs were characterized by tachypnoea (peak 109 breaths per minute), labored abdominal breathing and occasionally dyspnoea. Mean respiratory scores at 3 and 6 days after challenge were 9.8 and 9.5, respectively. Infection-immune pigs (group A3), on the other hand, experienced only transient general signs upon challenge and LPS exposure. Respiratory signs were absent. Clinical scores of these pigs were significantly lower than those of non-immunized challenged pigs ($P<0.05$). In pigs immunized with

the attenuated American strain (group A1), LPS administrations at 3 and 6 days after challenge induced severe general and respiratory signs in all pigs. General signs were characterized by fever (peak 42.1°C) and depression. Respiratory signs were characterized by tachypnoea (peak 123 breaths per minute), abdominal breathing and occasionally dyspnoea. Mean respiratory scores at 3 and 6 days after challenge (10.8 and 12.3, respectively) were similar to those of non-immunized challenged pigs ($P>0.05$). In pigs immunized with the attenuated European strain (group A2), LPS induced mainly transient general signs. Respiratory signs were minimal or absent. Mean respiratory scores at 3 and 6 days after challenge (0.0 and 0.3, respectively) were significantly lower than those of non-immunized challenged pigs ($P<0.05$).

Experiment B - In non-immunized challenged pigs (group B4), LPS induced again severe general and respiratory signs in all pigs. General signs were characterized by fever (peak 41.8°C) and severe depression. Pigs huddled together while showing signs of chilling, listlessness and inappetence. Respiratory signs were characterized by tachypnoea (peak 116 breaths per minute), labored abdominal breathing and dyspnoea. Mean respiratory scores at 3 and 6 days after challenge were 14.8 and 13.0, respectively. Infection-immune pigs (group B3), on the other hand, experienced very mild general and respiratory signs upon challenge and LPS exposure. Clinical scores of these pigs were clearly lower than those of non-immunized challenged pigs. In pigs immunized with the attenuated American strain (group B1), LPS administrations at 3 and 6 days after challenge induced moderate to severe clinical signs. All pigs exhibited fever (peak 41.4°C) and depression. Respiratory signs were observed in 80% of the pigs. There was tachypnoea (peak 96 breaths per minute), abdominal breathing and occasionally dyspnoea. The mean respiratory score at 3 days after challenge (4.0) was significantly lower than that of non-immunized challenged pigs ($P<0.05$), whereas the mean respiratory score at 6 days after challenge (7.6) did not differ from that of non-immunized challenged pigs ($P>0.05$). In pigs immunized with the attenuated European strain (group B2), LPS induced general and respiratory signs in all pigs. General signs included fever (peak 41.4°C) and depression. Respiratory signs were characterized by tachypnoea (peak 120 breaths per minute) and abdominal breathing. Mean respiratory scores at 3 and 6 days after challenge (8.7 and 6.6, respectively) did not differ from those of non-immunized challenged pigs ($P>0.05$).

Virus titration of BAL fluids and lung tissue. The results of virus titrations of BAL fluids and lung tissue of the individual pigs of the different groups, euthanized at 4 and 7 days after intratracheal (experiment A) or intranasal (experiment B) Lelystad virus challenge are shown in Figure 1. Challenge virus was isolated from BAL fluids and lung tissue of all non-immunized challenge control pigs (groups A4 and B4) at both times examined. Virus titres in BAL fluids and lung tissue were similar. When the challenge virus was inoculated by intratracheal route (group A4), the highest virus titres were reached at 4 days after challenge ($10^{6.2}$ $TCID_{50}$ per ml BAL fluid and per gram lung tissue). When the challenge virus was inoculated by intranasal route (group B4), the highest virus titres were reached at 7 days after challenge ($10^{6.2}$ $TCID_{50}$ per ml BAL fluid and per gram lung tissue).

Upon intratracheal challenge, two out of two (4 days after challenge) and one out of two (7 days after challenge) infection-immune pigs (group A3) were virus-positive. Their mean virus titres ($10^{2.0}$ $TCID_{50}$ per gram lung tissue) were significantly lower than those of the non-immunized challenge control pigs ($P<0.05$). Upon intranasal challenge, all four infection-immune pigs (group B3) were virus-negative.

The two commercial vaccines reduced the number of virus-positive pigs and the virus titres to different degrees. In experiment A, all six pigs immunized with the attenuated American strain (group A1) were virus-positive. The mean virus titres at 4 days after challenge ($10^{5.9}$ and $10^{6.4}$ $TCID_{50}$ per ml BAL fluid and per gram lung tissue, respectively) did not differ significantly from those of the non-immunized challenge control pigs ($P>0.05$). The mean virus titres at 7 days after challenge ($10^{3.4}$ and $10^{4.5}$ $TCID_{50}$ per ml BAL fluid and per gram lung tissue, respectively) were significantly lower than those of the non-immunized challenge control pigs ($P<0.05$). In the group immunized with the attenuated European strain (group A2), all six pigs tested positive for virus in the BAL fluids. The three pigs, euthanized at 7 days after challenge, were positive for PRRSV in BAL fluids, but not in lung tissue. The mean virus titres in BAL fluids ($10^{3.8}$ and $10^{1.9}$ $TCID_{50}$ per ml BAL fluid at 4 and 7 days after challenge, respectively) and lung tissue ($10^{3.6}$ and $10^{0.9}$ $TCID_{50}$ per gram lung tissue at 4 and 7 days after challenge, respectively) were significantly lower than those of the non-immunized challenge control pigs ($P<0.05$).

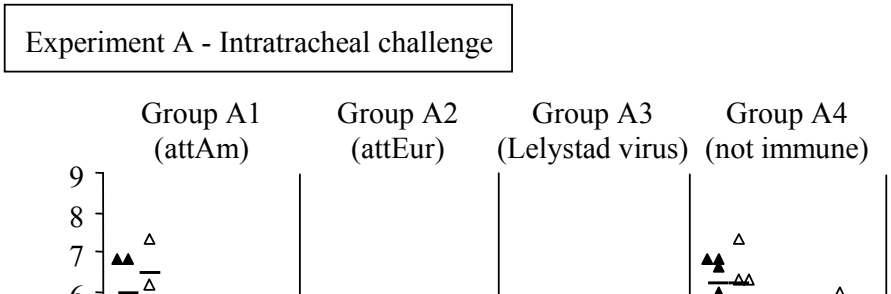

Fig. 1. Virus titres in broncho-alveolar lavage (BAL) fluids (log_{10} $TCID_{50}$/ml), lung tissue (log_{10} $TCID_{50}$/gram) and serum samples (log_{10} $TCID_{50}$/ml) at 4 and 7 days after intratracheal (experiment A) or intranasal (experiment B) challenge with Lelystad virus. Triangles represent individual virus titres in BAL fluids (σ) and lung tissue (Δ). Bullets represent individual virus titres in sera (O). Dashes represent group means at each time point.

attAm : attenuated American strain, attEur : attenuated European strain

In experiment B, nine out of the ten pigs immunized with the attenuated American strain (group B1) were virus-positive and the mean virus titres in BAL fluids ($10^{4.3}$ and $10^{6.8}$ $TCID_{50}$ per ml BAL fluid at 4 and 7 days after challenge, respectively) and lung tissue ($10^{4.9}$ and $10^{6.3}$ $TCID_{50}$ per gram lung tissue at 4 and 7 days after challenge, respectively) did not differ from those of the non-immunized challenge control pigs

(P>0.05). In the group immunized with the attenuated European strain (group B2), virus was isolated from only three out of the five pigs at 4 days after challenge and from only two out of the five pigs at 7 days after challenge. The mean virus titre in lung tissue at 4 days after challenge ($10^{2.2}$ $TCID_{50}$ per gram lung tissue) was significantly lower than that of the non-immunized challenge control pigs (P<0.05), while the reduction in virus titre was just not significant in the BAL fluids ($10^{2.7}$ $TCID_{50}$ per ml BAL fluid) (P>0.05). At 7 days after challenge, the mean virus titres in BAL fluids and lung tissue ($10^{1.6}$ $TCID_{50}$ per ml BAL fluid and $10^{1.5}$ $TCID_{50}$ per gram lung tissue) were significantly lower than those of the non-immunized challenge control pigs (P<0.05).

Virus titration of serum samples. At the time of challenge, neither vaccine nor virulent virus was detected in serum of any of the pigs.

The results of virus titrations of serum samples of the individual pigs of the different groups, euthanized at 4 and 7 days after intratracheal (experiment A) or intranasal (experiment B) Lelystad virus challenge are shown in Figure 1. Challenge virus was isolated from the sera of all non-immunized challenge control pigs (groups A4 and B4), except for one pig euthanized at 7 days after intranasal challenge. All infection-immune pigs (groups A3 and B3) were virus-negative in their serum. Fourteen of the total of sixteen pigs immunized with the attenuated American strain were virus-positive in their serum. Their mean virus titres were similar to those of the non-immunized challenge control pigs (P>0.05), except at 7 days after intratracheal challenge (P<0.05). In contrast, only two of the total of sixteen pigs immunized with the attenuated European strain (groups A2 and B2) were virus-positive in their serum.

Discussion

To our knowledge, this is the first study on virological protection of the respiratory tract of pigs vaccinated with modified live PRRSV vaccines. Under the given experimental circumstances, vaccination against PRRSV provides only partial virological protection of the lungs. Protection against infection with the European Lelystad strain was better after vaccination with a European strain than with an antigenically and genetically more distant American strain. One of the most surprising results was that infection-immune pigs, which were immunized with virulent Lelystad virus and were challenged with the same virulent Lelystad strain, did not show a complete virological

protection in the lungs. Thus, even in this fully homologous situation, PRRSV is still able to replicate in the lungs.

The present experimental study shows that vaccines, whether based on American or European virus strains, are not able to afford a complete virological protection in the lungs. Thus, it is not likely that vaccination, even if extensively applied, will be able to drastically reduce or eliminate PRRSV circulation in the swine population. This may be particularly true if field strains are genetically and antigenically divergent from the vaccine strains. Such genetic diversity exists between American and European PRRSV isolates (Wensvoort et al., 1992; Bautista et al., 1993), but also appears to exist among European field isolates. A recent study has demonstrated that, in Western Europe, PRRSV field isolates show so much genetic diversity in their open reading frames 5 and 7 that three clusters were identified: a Lelystad-like cluster, a purely Danish cluster and a highly diverse Italian-like cluster (Forsberg et al., 2002). These findings may have important consequences with regard to vaccine efficacy and the selection of virus strains for vaccine purposes. Thus, a continuous update of vaccine strains may be necessary to reach an acceptable level of protection in the field, even within geographical areas of limited size. Possibly, PRRSV vaccines containing different genotypes may be needed in the future to compensate for such genetic diversity.

We compared intranasal and intatracheal challenge methods because we hypothesized that the challenge virus replication might be lower after intranasal than after intratracheal inoculation. However, the route of inoculation apparently did not affect the degree of challenge virus replication in the lungs. Our data indicate that replication of the challenge virus may be delayed after intranasal inoculation, but certainly not reduced, when compared with an intratracheal challenge.

Correlations between virus titres in the lungs and those in serum were significant at group level, but not in the individual pigs. Eleven of the total of thirty-two vaccinated pigs were negative for challenge virus in their sera, whereas their BAL fluids and/or lung tissue were positive. Duan et al. (1997) obtained similar results in naive pigs inoculated with PRRSV. In their study, five of twelve pigs examined at $\geq$ 21 days post inoculation had PRRSV-negative sera, while they were still virus-positive in the lungs. All these results demonstrate that efficacy tests of modified live PRRSV vaccines should not be based on virus titres in blood alone, but should also focus on the degree of virus replication in the lungs upon challenge. Based on the present data, we recommend to perform virus titrations on both BAL fluids and lung tissue. Pigs with high PRRSV titres

had positive BAL fluids and lungs, but this was not always the case for pigs with low titres ($\leq 10^{3.3}$ $TCID_{50}$). Lung samples of six of the total of twenty-four pigs immunized with a European strain were negative for challenge virus, whereas BAL fluids were virus-positive, or vice versa.

The present experiments were also undertaken to examine if the challenge virus replication in the lungs of vaccinated pigs predisposes for respiratory signs upon LPS exposure. Singly LPS-exposed pigs were not included because of the limited number of pigs available for these experiments. Earlier experiments had demonstrated that singly LPS-exposed pigs developed transient general signs and no, or only mild respiratory signs (Labarque et al., 2002). Respiratory signs were mild or absent in infection-immune pigs. Non-immunized challenged pigs, on the other hand, suffered from severe respiratory signs upon LPS exposure, such as tachypnoea, abdominal breathing and dyspnoea. In contrast, the clinical outcome upon LPS exposure of pigs that had been vaccinated and six weeks later challenged with PRRSV was highly variable within and between experiments. Pigs immunized with the attenuated European strain experienced no respiratory signs in experiment A, whereas they developed clear respiratory signs in experiment B. Similarly, all pigs immunized with the attenuated American strain developed severe respiratory signs upon LPS exposure in experiment A, whereas in experiment B, respiratory signs were milder and similar to those of the pigs immunized with the attenuated European strain. Thus, we were unable to reproduce the clinical results in two subsequent attempts. The severity of the clinical signs upon LPS exposure was not correlated with the degree of virus replication in the lungs of individual pigs. Thus, it remains unclear why clinical synergy occurs reproducibly in PRRSV-infected pigs, but not in PRRSV-vaccinated and challenged pigs even though challenge virus replication occurs.

Taken together, the present data show that the virological protection in the lungs of infection- and vaccination-immune pigs upon challenge is incomplete, but is more pronounced in the homologous situation. The variable clinical outcome upon LPS exposure of vaccinated pigs hampers the use of the combined PRRSV-LPS exposure for testing of the clinical efficacy of modified live PRRSV vaccines.

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6.

GENERAL DISCUSSION

General discussion

It was shown in the present studies that two stages can be considered during a porcine reproductive and respiratory syndrome virus (PRRSV) infection in the lungs of pigs: an acute stage comprising the first two weeks of infection during which high amounts of virus are present, and a persistent stage of 3 to 4 weeks characterized by lower levels of virus replication. Virus replication was highest at 7 to 9 days post inoculation (PI) and decreased thereafter. The number of PRRSV-infected cells in lung tissue and in broncho-alveolar spaces showed a marked decrease after 9 days PI. Our data suggest that this decrease is the result of the appearance of anti-PRRSV antibodies in the lungs and major changes in the population of well-differentiated macrophages, the PRRSV target cells (Duan et al., 1997b), leading to depletion of susceptible cells. A low number of PRRSV-infected cells was able to persist in lungs and broncho-alveolar lavage (BAL) fluids until 35 to 40 days PI. This persistence may be explained by the late appearance of immune mechanisms capable of eliminating the virus, i.e. virus-neutralizing antibodies and virus-specific T lymphocytes. Antibodies generated during the early phase of infection were non-neutralizing. Though non-neutralizing antibodies can mediate the killing of infected cells by complement or by cells of the innate immune system, virus-neutralizing antibodies are generally thought to be more efficient in virus clearance. Such virus-neutralizing antibodies appeared much later and were only detected starting from 25 days PI in the present study and also in other studies (Yoon et al., 1995; Albina et al., 1998). Further, other researchers reported that PRRSV-specific T lymphocytes were only detected starting from 28 days PI (Bautista & Molitor, 1997; López-Fuertes et al., 1999). Why the PRRSV-specific immune response is characterized by an unusual delay of some arms of the humoral and cellular immune response is not yet known. Meier et al. (2000) suggested that cytokines such as interleukin (IL)-10 may be responsible for the suppression of the cellular immune response against PRRSV. The latter hypothesis is supported by our studies, since IL-10 was found in BAL fluids during a prolonged period, i.e. from 5 until 25 days PI.

The virus persistence in individual pigs has important epidemiological consequences. PRRSV-infected pigs function as long-term virus excretors transmitting the virus to susceptible contact pigs for long periods of time. Thus, PRRSV persists at herd and population level which makes control and/or elimination of PRRSV difficult. The virus

persistence also hampers a proper diagnosis of respiratory disease problems in nursery and fattening pigs. In swine herds with respiratory disease problems, PRRSV is often incriminated as the causative agent based on a positive isolation from the lungs. However, this diagnosis should be interpreted with caution since PRRSV may not be of major significance in causing respiratory disease problems during the persistent stage of the infection.

The present studies revealed important cellular changes in the lungs throughout a PRRSV infection, especially in the population of monocytes/macrophages. The total number of broncho-alveolar monocytes/macrophages markedly increased from 5 until 52 days PI with a maximum at 25 days PI. Also, the composition of the population of monocytes/macrophages showed important shifts throughout the infection. It was demonstrated that susceptible well-differentiated pulmonary macrophages, carrying the PRRSV receptor, became destroyed, either by cell lysis due to virus replication or by apoptosis, and were replaced by blood monocytes. With time, these blood monocytes differentiated into macrophages as demonstrated by morphological changes and expression of the PRRSV receptor. The rather specific influx of monocytes is indicative of a very selective chemotactic signal. During this massive influx of blood monocytes, the total number of monocytes/macrophages in the lungs appears to be partially regulated by apoptosis. This was indicated by the observation that high numbers of interstital monocytes/macrophages underwent apoptosis during the time period in which blood monocytes entered the broncho-alveolar spaces. However, the process of apoptosis was apparently not sufficient to control the massive influx of blood monocytes since abundant monocytes/macrophages crossed the interstitium towards the broncho-alveolar spaces. The occurrence of apoptosis in the lungs of PRRSV-infected pigs may also explain why only a very mild lung inflammation (Pol et al., 1991) with low percentages of neutrophils (Van Reeth et al., 1999) is present during a PRRSV infection. A general feature of apoptotic cell death is that apoptotic bodies are phagocytised by resident macrophages without provoking an inflammatory response.

A single PRRSV infection, particularly under experimental circumstances and with European isolates, fails to induce overt respiratory signs (Plana-Durán et al., 1992; Ramos et al., 1992; Van Reeth et al., 1996). Also under field circumstances, most pigs become infected with PRRSV without respiratory disease (Houben et al., 1995). Still, the frequency and severity of respiratory disease problems in the field have increased since the enzootic occurrence of PRRSV (Done & Paton, 1995). In herds with respiratory

disease problems, PRRSV has been isolated in combination with several bacteria and viruses. These observations have stimulated research into the combined effects of PRRSV and other pathogens. Consequently, experimental dual infections with PRRSV followed by various bacteria and viruses have been performed. Variation in the severity of clinical signs and lack of reproducibility were the main problems with these experimental dual infections. Even single experimental infections with respiratory viruses show variation in clinical and inflammatory parameters. A second infection with a virus or bacterium may enhance this variation. Therefore, we have performed experiments in which PRRSV-infected pigs were 5 days later exposed to a non-replicating agent, namely lipopolysaccharide (LPS). LPS was chosen because of epidemiological and pathogenetic reasons. LPS are present in varying concentrations in dust in swine confinement units (Rylander, 1994; Zejda et al., 1994; Zhiping et al., 1996) and are released in the lungs upon infection and colonization with Gram-negative bacteria (Pugin et al., 1992). Further, LPS is known to stimulate cells of the monocyte/macrophage lineage and our pathogenesis studies have shown important cellular changes in this cell population. The PRRSV-LPS combination induced clear respiratory signs in 90% of the pigs and was reproducible within and between experiments.

Respiratory signs as a result of the synergy between PRRSV and LPS could not be explained by an enhancement of inflammatory changes in the lungs. The finding that inflammatory cell profiles in the BAL fluids of PRRSV-LPS exposed pigs were merely an accumulation of those observed with PRRSV only and LPS only suggests that inflammatory changes in the lungs are not responsible for the clinical synergy between PRRSV and LPS. Functional lung changes such as bronchial hyper-responsiveness appear to be responsible for the observed respiratory signs. Van Gucht et al. (2002) demonstrated that respiratory signs were clearly associated with levels of pro-inflammatory cytokines. Peak tumour necrosis factor-alpha (TNF-α) and IL-1 titres were 10 to 100 times higher in PRRSV-LPS exposed pigs than in PRRSV and LPS control pigs and they correlated with respiratory signs. Interestingly, both TNF-α and IL-1 have been demonstrated to cause bronchial hyper-responsiveness (Kips et al., 1992; Thomas et al., 1995), leading to an increased and sustained bronchoconstriction. Taken together, the production of TNF-α and IL-1 in PRRSV-LPS exposed pigs will likely play a role in the induction of respiratory signs. Similar findings were made in a previous experimental

study, in which porcine respiratory coronavirus-infected pigs were exposed to LPS (Van Reeth et al., 2000). The mechanisms whereby PRRSV can trigger the lungs for an enhanced cytokine production upon LPS exposure remain to be defined. Cells of the monocyte/macrophage lineage are the main target cells for LPS and they are potent producers of the pro-inflammatory cytokines TNF-α and IL-1. Thus, it seems logical that the massive influx of new monocytes in the lungs, as shown in our pathogenesis studies, may contribute to an enhanced response to LPS.

The PRRSV-LPS combination seems to be relevant under field circumstances. All pigs become infected with PRRSV at ages varying from 4 weeks to fattening age (Albina et al., 1994; Houben et al., 1995; Mateusen et al., 2002) and PRRSV persists in the lungs for 35 to 49 days after inoculation (Mengeling et al., 1995; Duan et al., 1997a; Labarque et al., 2000a). Thus, there is a long time period during which the lungs of PRRSV-infected pigs may become exposed to airborne or locally produced LPS. Still, the true significance of the PRRSV-LPS combination for multi-factorial respiratory disease problems in the field remains to be defined. In our experimental studies, LPS was administered to pigs at a dose of 20 μg/kg body weight. This dose was used because previous studies had shown that this dose caused mild or no respiratory signs in naive pigs (Van Reeth et al., 2000). It is difficult to calculate the daily exposure to LPS under field circumstances. Assuming a respiratory volume of 0.3 m^3/hour in an environment with an endotoxin concentration of 5 μg/m^3 air (Zhiping et al., 1996), pigs of the same age as in our experimental model would be exposed to a total dose of airborne endotoxins of approximately 36 μg per day. The amount of LPS which is released in the lungs upon infection with Gram-negative bacteria in pigs has not been measured until now. In humans, Pugin et al. (1992) demonstrated endotoxin concentrations of 0.375 μg in BAL fluids of one lung lobe of patients with a severe Gram-negative bacterial pneumonia. Thus, it seems that pigs in the field are exposed to lower doses of LPS than those used experimentally. Moreover, LPS tolerance can be induced by continuous LPS exposure. Naive humans exposed acutely to swine confinement buildings display more intense lung functional and inflammatory changes than workers exposed chronically to this environment (Larsson et al., 1994; Cormier et al., 1997). Experimental evidence of LPS tolerance has already been demonstrated in laboratory animals, which were daily exposed to LPS during 15 minutes (Shimada et al., 2000). In conclusion, the relevance of the PRRSV-LPS combination to the field situation needs further investigation. Nevertheless,

the PRRSV-LPS combination has been proven to be reproducible and straightforward under experimental circumstances. It is one of the few combined inoculations in pigs that has proven to consistently induce respiratory signs. Therefore, we believe that this combination is appropriate for the study of the pathogenesis of multi-factorial respiratory disease problems.

Multi-factorial respiratory disease problems remain an economically important problem in swine production worldwide. Since PRRSV is an important contributor to these problems (Done & Paton, 1995), several modified live PRRSV vaccines have been developed and licensed for use in feeder pigs. Important safety and efficacy concerns have been addressed concerning these vaccines.

A major safety concern relating to modified live vaccines is the dissemination of the vaccine virus throughout the body after vaccination. The presence of vaccine virus in blood has been documented by several research groups (Christopher-Hennings et al., 1996; Bøtner et al., 1997; Stadejek & Pejsak, 1998; Astrup & Riising, 2002), but information about the replication of vaccine viruses in target organs of PRRSV, such as lymphoid organs and lungs, is scarce. Our studies demonstrated that vaccine virus strains are able to replicate in the lungs for at least 12 days after intratracheal inoculation. Based on these findings, it is possible that vaccine virus replication in the lungs may predispose for the appearance of multi-factorial respiratory disease problems under field circumstances. In this regard, it was shown in the present studies that vaccine viruses can sensitize the lungs for respiratory signs upon LPS exposure, but the clinical signs were less severe and of shorter duration than with the virulent Lelystad strain.

Efficacy tests for modified live PRRSV vaccines are nowadays based on the degree of reduction in viraemia (van Woensel et al., 1998; Labarque et al., 2000b). Until now, no information was available about the degree of virological protection in the lungs, the main target organ of the virus. In order to address this question, pigs were vaccinated with a modified live PRRSV vaccine, either based on a European or an American virus strain. The challenge was performed with a European wild-type strain 6 weeks later, and pigs were euthanized at 4 and 7 days after challenge for virological examinations of lungs and sera. Challenge virus replication in the lungs still occurred in both vaccinated groups, but the virological protection was better in pigs vaccinated with the European serotype vaccine compared to pigs vaccinated with the American serotype vaccine. Thus, the antigenic and genetic differences between European and American PRRSV strains (Wensvoort et al., 1992; Bautista et al., 1993) clearly affect the degree of cross-protection

in the lungs. However, even infection-immune pigs, which were infected with virulent virus, built up antibodies and were challenged six weeks later with the same virulent PRRSV strain, did not show a complete virological protection in the lungs. Although the mechanisms that mediate protective immunity against PRRSV are unknown, it is reasonable to suggest that the late appearance of both neutralizing antibodies and virus-specific T lymphocytes following either a PRRSV wild-type infection or vaccination is responsible for the incomplete virological protection in the lungs. The development of more effective PRRSV vaccines will require a better understanding of the mechanisms that regulate the humoral and cellular immune response against this virus. Since it has been demonstrated that the clearance of PRRSV from the lungs coincides with the appearance of both neutralizing antibodies and virus-specific T lymphocytes, stimulation of these immune components by vaccination may enhance vaccine efficacy. In theory, it is possible to locate and define the epitopes on viral proteins that elicit neutralizing antibodies or that are recognized by T lymphocytes. Artificial constructs of such epitopes, so-called synthetic peptide vaccines, have already been used for vaccination against other viral diseases. Further, it is worth examining whether cytokines such as IL-10 may hamper the cellular immune response against PRRSV and whether anti-cytokine strategies can solve this problem.

The present virological data may have some practical consequences with regard to vaccination under field circumstances. Our findings demonstrate that if vaccinated pigs become infected with wild-type virus, this virus still replicates in the lungs of these pigs. Thus, it is likely that this virus will be able to spread substantially among pigs. However, caution should be taken to extrapolate the results of these vaccination-challenge experiments to effectiveness of vaccination under field conditions. In the present experiments, pigs were challenged with a very high dose of virulent virus and it is unlikely that pigs will encounter such high virus doses under field conditions.

The incomplete virological protection in the lungs also raises the question whether the field virus replication in the lungs of vaccinated pigs may predispose for multi-factorial respiratory disease problems under field circumstances. The LPS administration was used to examine that aspect. Vaccinated and subsequently challenged pigs were exposed to LPS 3 and 6 days after challenge. The clinical outcome upon LPS exposure was highly variable within and between two subsequent experiments.

Although correlations between virological results in lungs and viraemia were significant at group level, important differences were found at individual level. Serum

samples of a considerable number of vaccinated pigs were negative for challenge virus, whereas their corresponding BAL fluids and/or lung samples were virus-positive. The latter finding was supported by Duan et al. (1997a), who showed that PRRSV-infected pigs became virus-negative more rapidly in serum samples than in lung tissue. Thus, viraemia parameters are not an accurate indicator of the virological protection in vaccinated pigs and efficacy tests should not be based on virus titres in blood alone, but should also focus on the degree of virus replication in the lungs upon challenge.

In conclusion, the present studies have shown that the safety and efficacy of the current modified live PRRSV vaccines are not that satisfactory under experimental circumstances. A better understanding of the mechanisms that regulate the immunity against PRRSV will be essential for the development of more effective vaccines. Based on the findings of the present studies, it is recommended that safety and efficacy assays for PRRSV vaccine candidates should focus on the main target organ of the virus, the lungs.

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7.

SUMMARY / SAMENVATTING

Summary

Respiratory disease causes worldwide important financial losses in swine husbandry. This disease has a multi-factorial background and porcine reproductive and respiratory syndrome virus (PRRSV) is generally recognized as one of the major infectious agents involved in this disease.

The aims of this thesis were (i) to extend the knowledge on the pathogenesis of a PRRSV infection in the lungs, (ii) to investigate the interaction between PRRSV and lipopolysaccharide (LPS) and (iii) to assess the efficacy of modified live PRRSV vaccines using virological and clinical parameters of protection.

In chapter 1, a review was given on PRRSV with special attention to the pathogenesis, clinical disease and vaccination. Further, a brief introduction was given about lipopolysaccharides or endotoxins and their role in respiratory disease problems.

In chapter 3, the pathogenesis of a single PRRSV infection in the lungs was studied with a European strain. Special attention was given to cellular changes in lung tissue and broncho-alveolar lavage (BAL) cells and these events were correlated with virus replication and appearance of neutralizing and non-neutralizing antibodies.

In part 3.1., a number of pathogenetic events in the lungs of PRRSV-infected gnotobiotic pigs were studied in detail with the purpose to relate the degree and pattern of virus replication with cellular changes and the onset of PRRSV-specific antibodies. Four- to five-week-old gnotobiotic pigs were inoculated intranasally with the Lelystad strain of PRRSV and euthanized between 1 and 52 days post inoculation (PI). The BAL cell population was morphologically and phenotypically characterized, together with the pattern and degree of virus replication and the appearance of non-neutralizing and neutralizing antibodies. The total number of monocytes/macrophages increased two- to fivefold between 9 and 52 days PI with a maximum at 25 days PI. Flow cytometric analysis showed that the population of well-differentiated macrophages that express the PRRSV receptor (74-22-15$^+$, 41D3$^+$ cells) was reduced between 9 and 20 days PI and that between the same time interval small 74-22-15$^+$, 41D3$^-$ cells, presumably blood monocytes, and small 74-22-15$^-$, 41D3$^-$ cells, presumably lymphocytes, entered the alveolar spaces. Virus replication peaked at 7 to 9 days PI, decreased slowly thereafter and was detected until 40 days PI. PRRSV-specific antibodies were detected in BAL fluid starting at 9 days PI, whereas specific neutralizing antibodies were only

demonstrated in two pigs euthanized at the end of the study, at 35 and 52 days PI. The decrease of virus replication in the lungs from 9 days PI can be attributed to (i) shortage of susceptible well-differentiated macrophages, (ii) lack of susceptibility of the newly infiltrated blood monocytes and (iii) appearance of PRRSV-specific antibodies in the lungs. Neutralizing antibodies may contribute to the complete elimination of virus from the lungs.

In part 3.2., the kinetics of apoptosis in the lungs were investigated with a European strain of PRRSV and it was examined if cytokines are involved in the induction of apoptosis. Also, an attempt was made to clarify a possible role of apoptosis in the pathogenesis. Lungs and BAL cells of the pigs, that had been used in part 3.1., were assessed both for virus replication and apoptosis, whereas BAL fluids were examined for interleukin (IL)-1, tumour necrosis factor-alpha (TNF-α) and IL-10 production. Double-labeling experiments were conducted to determine the relation between virus replication and apoptosis and for phenotypical identification of the apoptotic cells. Apoptosis was detected in both infected and non-infected cells. The mean percentages of infected cells, which were apoptotic, ranged between 9 and 39% in lung tissue and between 13 and 30% in BAL cells. The majority of apoptotic cells (>99%) were, however, non-infected. The pattern of apoptosis in the non-infected cells was different in the broncho-alveolar spaces compared to lung tissue. In the broncho-alveolar spaces, two peaks of apoptosis were detected. The first peak at 9 days PI involved mainly lymphocytes. The second peak of apoptosis at 25 days PI can be explained by the fact that the total BAL cell numbers were the highest at that time point. In lung tissue, apoptosis peaked at 14 days PI and occurred predominantly in monocytes/macrophages. The peak of apoptosis in lung tissue was preceded by a peak in IL-1 and IL-10 production in BAL fluids at 9 days PI, suggesting a possible role of these both cytokines in the induction of apoptosis in non-infected interstitial monocytes/macrophages. The latter hypothesis was, however, not confirmed by *in vitro* studies, since blood monocytes or alveolar macrophages did not undergo apoptosis upon treatment with recombinant porcine IL-1 or IL-10. Thus, the exact mechanism by which PRRSV triggers apoptosis in non-infected interstitial monocytes/macrophages is still under debate.

Based on the results, obtained in parts 3.1. and 3.2., the following hypothetical roles of apoptosis in the pathogenesis of a PRRSV infection in the lungs can be made. Apoptosis of infected cells may be one of the explanations for the persistence of single PRRSV-infected cells until 35 to 40 days PI, despite the presence of PRRSV-specific antibodies.

Apoptosis of non-infected interstitial monocytes/macrophages may be a process to regulate the number of monocytes/macrophages during the massive influx of monocytes in the lungs.

In chapter 4, the interaction between PRRSV and LPS was examined. The clinical course of inoculations with PRRSV followed by LPS, and the effect of the timing and frequency of LPS administrations were examined. Furthermore, it was examined if the virulence of the PRRSV strain affects the interaction.

In part 4.1., the clinical effect of an exposure with *Escherichia coli* LPS in PRRSV-infected pigs was examined. Additionally, some preliminary investigations of cellular and virological aspects in the lungs were performed. Five-week-old conventional pigs were inoculated intratracheally with the Lelystad strain of PRRSV and received 5 days later one or two LPS administrations by intratracheal route. Control groups consisted of pigs inoculated with PRRSV only or LPS only. All pigs were intensively monitored for clinical signs after the LPS administrations. Pigs were monitored for general (fever and depression) and respiratory (tachypnoea, abdominal breathing and dyspnoea) signs. Some pigs were euthanized after the second LPS administration for broncho-alveolar cell analysis and virological examinations of the lungs. PRRSV-LPS exposed pigs developed clear respiratory signs, characterized by tachypnoea, abdominal breathing and dyspnoea. This was in contrast to PRRSV and LPS control pigs, which developed no or only very mild respiratory signs. Most PRRSV and LPS control pigs developed moderate fever, but no depression. PRRSV-LPS exposed pigs, on the other hand, developed high fever and typically showed pronounced depression. The clinical effect of a second LPS administration was dependent on the time interval between the two LPS administrations. In non-infected pigs, a second LPS administration at a 24-hour interval caused milder clinical signs than the first one. On the other hand, a second LPS administration within a 3-hour interval seriously aggravated and prolonged the clinical signs. In PRRSV-infected pigs, the clinical effect of a second LPS administration was difficult to assess since pigs had not yet recovered at the moment of the second LPS administration, 3 or 24 hours after the first one. Lung neutrophil infiltration was similar in non-infected and PRRSV-infected pigs upon LPS exposure. PRRSV titres in lung tissue and BAL fluids were similar in PRRSV control and PRRSV-LPS exposed pigs. Thus, the respiratory signs following PRRSV-LPS exposure could not be explained by the degree of virus replication or inflammatory changes in the lungs. In conclusion, this study showed a clear synergy between PRRSV and LPS in the induction of respiratory signs in

conventional pigs. The synergy was observed in 87% of the pigs and was reproducible upon subsequent experiments.

In part 4.2., it was examined if there exists also a clinical synergy between attenuated PRRSV strains and LPS. Two experiments were performed. In each experiment, five-week-old conventional pigs were inoculated intratracheally with a commercial modified live PRRSV vaccine, based on either an attenuated European virus strain or an attenuated American virus strain. Pigs inoculated intratracheally with Lelystad virus and non-inoculated pigs were included as controls. At 5 days after virus inoculation, pigs were exposed to *Escherichia coli* LPS by intratracheal route. All pigs were followed clinically every two hours from 0 until 12 hours after the LPS administration. As in part 4.1., pigs were monitored for general (fever and depression) and respiratory (tachypnoea, abdominal breathing and dyspnoea) signs. Additionally, pigs of the second experiment were euthanized at 12 hours after the LPS administration for virological examinations of lungs and BAL fluids (virus titration and quantification of infected cells). Exposure of non-inoculated pigs to LPS resulted in slight to moderate respiratory signs. Lelystad virus-LPS exposed pigs experienced severe respiratory signs, characterized by tachypnoea, abdominal thumping and dyspnoea. Respiratory signs of vaccine virus-LPS exposed pigs were less pronounced and of shorter duration than those of Lelystad virus-LPS exposed pigs. The differences between virulent virus- and vaccine virus-inoculated pigs may be attributed to the pattern and degree of virus replication in the lungs. Mean virus titres and mean numbers of infected cells were indeed clearly higher in virulent virus-inoculated pigs compared to vaccine virus-inoculated ones. Furthermore, viral antigen-positive foci, consisting of groups of three or more infected cells and cellular debris, were only observed in the lung tissue of virulent virus-inoculated pigs. In summary, the present study shows that there exists a clinical synergy between attenuated PRRSV strains and LPS, but the severity is less pronounced than with the virulent Lelystad strain.

In chapter 5, strategies to prevent multi-factorial respiratory disease problems were examined by evaluating the virological and clinical protection provided by modified live PRRSV vaccines. The virological protection was assessed in the lungs, the main target organ of PRRSV. To our knowledge, this was the first study demonstrating the degree of virological protection in the lungs of PRRSV-vaccinated pigs. For the evaluation of the clinical efficacy of PRRSV vaccines, the PRRSV-LPS combination was used. Two experiments were performed. In each experiment, six-week-old conventional pigs were

immunized intramuscularly with commercial vaccines, based on either an attenuated European virus strain or an attenuated American virus strain. Non-immunized pigs and pigs immunized intramuscularly with the virulent Lelystad strain (infection-immune pigs) were included as controls. Six weeks after immunization, pigs were challenged intratracheally (exp. 1) or intranasally (exp. 2) with the European Lelystad strain, and 3 and 6 days later exposed to *Escherichia coli* LPS by intratracheal route. After the LPS administration, pigs were monitored for clinical signs. At 4 and 7 days after challenge, some pigs were euthanized for virological examinations of lungs. Challenge virus replication in the lungs still occurred in both vaccinated groups, but the virological protection was better in pigs immunized with the attenuated European strain compared to pigs immunized with the attenuated American strain. Thus, the genetic and antigenic differences between European and American PRRSV strains clearly affected the degree of virological protection in the lungs. Even infection-immune pigs did not show a complete virological protection in the lungs. After LPS exposure, none or only very mild respiratory signs were observed in the infection-immune pigs. Non-immunized challenge control pigs, on the other hand, suffered from severe respiratory signs upon LPS exposure. The clinical outcome in pigs immunized with the attenuated virus strains was highly variable within and between experiments. Pigs immunized with the attenuated European strain experienced no respiratory signs in experiment 1, whereas in experiment 2, all pigs suffered from severe respiratory signs. Similarly, all pigs immunized with the attenuated American strain in the first experiment developed severe respiratory signs upon LPS exposure, whereas in experiment 2, respiratory signs were less pronounced and restricted to 80% of the pigs. Thus, we were unable to reproduce our clinical results upon two subsequent attempts and therefore, we were unable to make conclusions about the clinical protection provided by modified live PRRSV vaccines.

As a general conclusion, it can be stated that a single PRRSV infection induces multiple marked changes in the lungs even though respiratory signs remain absent. It was further demonstrated that apoptosis, either as a cause or as a result, may be associated with these cellular changes.

With the PRRSV-LPS combination, it was clearly demonstrated that a PRRSV infection is able to sensitize the lungs for respiratory signs upon exposure to LPS. In contrast to previous dual inoculation experiments with PRRSV followed by other viruses and/or bacteria, the PRRSV-LPS combination has been proven to induce reproducible

respiratory disease. Therefore, we believe that this combination is a good model to study the pathogenesis of multi-factorial respiratory disease problems.

Our vaccination/challenge experiments clearly demonstrated that the virological protection in the lungs of PRRSV-vaccinated pigs is only partial. Thus, it cannot be excluded that the challenge virus replication in the lungs of vaccinated pigs might still predispose for multi-factorial respiratory disease problems.

Samenvatting

Ademhalingsproblemen veroorzaken wereldwijd zware economische verliezen in de varkenssector. Deze ademhalingsproblematiek heeft een multifactoriële achtergrond en het wordt algemeen aanvaard dat het porcien reproductief en respiratoir syndroom virus (PRRSV) een belangrijke rol speelt in het ontstaan van deze problemen.

De doelstellingen van deze thesis bestonden erin om (i) de kennis omtrent de pathogenese van een PRRSV infectie in de longen uit te breiden, (ii) de interactie tussen PRRSV en lipopolysacchariden (LPS) te onderzoeken en (iii) de werkzaamheid van levend verzwakte PRRSV vaccins te beoordelen aan de hand van virologische en klinische parameters.

In hoofdstuk 1 werd een overzicht gegeven over PRRSV met speciale aandacht voor de pathogenese, het ziektebeeld en de vaccinatie. Verder werd een korte inleiding gegeven over lipopolysacchariden of endotoxinen en hun rol in ademhalingsproblemen.

In hoofdstuk 3 werd de pathogenese van een infectie met een Europese PRRSV stam in de longen onderzocht. Speciale aandacht werd geschonken aan cellulaire veranderingen in het longinterstitium en de broncho-alveolaire ruimten en deze veranderingen werden gecorreleerd met de virusvermeerdering en het verschijnen van neutraliserende en niet-neutraliserende antistoffen.

In deel 3.1. werden een aantal pathogenetische gebeurtenissen in de longen van PRRSV-geïnfecteerde gnotobiotische biggen in detail bestudeerd met de bedoeling om correlaties te vinden tussen de virusvermeerdering enerzijds en bepaalde cellulaire veranderingen en het opkomen van PRRSV-specifieke antistoffen anderzijds. Hiertoe werden gnotobiotische biggen op 4 tot 5 weken leeftijd intranasaal geïnoculeerd met Lelystad virus en 1 tot 52 dagen later geëuthanaseerd. De broncho-alveolaire cellen werden morfologisch en fenotypisch gekarakteriseerd. Verder werden de graad en het patroon van de virusvermeerdering en het opkomen van neutraliserende en niet-neutraliserende antistoffen bestudeerd. Het totaal aantal monocyten/macrofagen steeg met een factor twee tot vijf tussen 9 en 52 dagen na inoculatie met een maximaal aantal op 25 dagen na inoculatie. Flowcytometrisch onderzoek toonde aan dat de populatie sterk-gedifferentieerde alveolaire macrofagen, die de PRRSV receptor tot expressie brengen (74-22-15$^+$, 41D3$^+$ cellen), sterk gereduceerd was tussen 9 en 20 dagen na inoculatie. Gedurende ditzelfde tijdsinterval werd een massale influx van zowel 74-22-

15^+, $41D3^-$ cellen, vermoedelijk bloedmonocyten, als van $74\text{-}22\text{-}15^-$, $41D3^-$ cellen, vermoedelijk lymfocyten, waargenomen. De virusvermeedering was maximaal op 7 tot 9 dagen na inoculatie, nam daarna geleidelijk af en werd waargenomen tot 40 dagen na inoculatie. PRRSV-specifieke antistoffen in de broncho-alveolaire ruimten werden het eerst waargenomen vanaf 9 dagen na inoculatie, maar neutraliserende antistoffen werden uitsluitend waargenomen bij twee dieren, die geëuthanaseerd werden op het einde van de studie, namelijk op 35 en 52 dagen na inoculatie. Het afnemen van de virusvermeerdering in de longen vanaf 9 dagen na inoculatie kan toegeschreven worden aan (i) het tekort aan gevoelige sterk-gedifferentieerde macrofagen, (ii) het niet gevoelig zijn van de nieuw aangetrokken monocyten en (iii) het verschijnen van PRRSV-specifieke antistoffen in de longen. Neutraliserende antistoffen zijn wellicht betrokken bij de uiteindelijke eliminatie van PRRSV uit de longen.

In deel 3.2. werd de kinetiek van apoptose in de longen van PRRSV-geïnfecteerde biggen bestudeerd met de bedoeling om een mogelijke rol van dit proces in de pathogenese te bepalen. Verder werd onderzocht of cytokinen betrokken zijn in de inductie van apoptose. Daartoe werden de longen en de broncho-alveolaire cellen van de dieren, die gebruikt werden in deel 3.1., onderzocht voor virusvermeerdering en apoptose en de overeenkomstige longwasvloeistoffen werden onderzocht voor de productie van interleukine (IL)-1, tumor necrosis factor-alfa (TNF-α) en IL-10. Dubbelkleuringen werden uitgevoerd om de relatie na te gaan tussen virusvermeerdering en apoptose en voor de fenotypische identificatie van de apoptotische cellen. Apoptose werd zowel waargenomen in geïnfecteerde cellen, als in niet-geïnfecteerde cellen. De gemiddelde percentages geïnfecteerde cellen, die apoptose vertoonden, varieerden tussen 9 en 39% in het interstitium en tussen 13 en 30% in de broncho-alveolaire ruimten. De meerderheid van de apoptotische cellen (>99%) waren echter niet geïnfecteerd. Het patroon van apoptose in de niet-geïnfecteerde cellen was verschillend in de broncho-alveolaire ruimten en het interstitium. In de broncho-alveolaire ruimten werden twee pieken van apoptose waargenomen. De eerste piek op 9 dagen na inoculatie betrof voornamelijk lymfocyten, terwijl de tweede piek op 25 dagen na inoculatie hoogstwaarschijnlijk te maken heeft met het feit dat het totaal aantal broncho-alveolaire cellen het hoogst was op dat tijdstip. In het interstitium werd de piek van apoptose waargenomen op 14 dagen na inoculatie en het waren voornamelijk monocyten/macrofagen, die apoptose vertoonden. De piek van apoptose in het interstitium werd voorafgegaan door een piek van IL-1 en IL-10 productie in de broncho-alveolaire ruimten op 9 dagen na inoculatie. Dit

suggereert dat beide cytokinen betrokken kunnen zijn in de inductie van apoptose in de niet-geïnfecteerde monocyten/macrofagen in het interstitium. Deze hypothese kon echter *in vitro* niet bevestigd worden, aangezien zowel bloedmonocyten als alveolaire macrofagen geen apoptose ondergingen na een behandeling met recombinant porcien IL-1 of IL-10. Het exact mechanisme waarop PRRSV apoptose induceert in niet-geïnfecteerde monocyten/macrofagen in het interstitium kon dus tot op heden niet achterhaald worden.

Op basis van de studies, zoals uitgevoerd in deel 3.1. en deel 3.2., kunnen de volgende pathogenetische aspecten in de longen van PRRSV-geïnfecteerde biggen eventueel toegeschreven worden aan apoptose. Het optreden van apoptose in geïnfecteerde cellen kan mogelijks één van de verklaringen zijn waarom PRRSV-geïnfecteerde cellen kunnen persisteren tot 35-40 dagen na inoculatie, ondanks de aanwezigheid van PRRSV-specifieke antistoffen. Het optreden van apoptose in niet-geïnfecteerde monocyten/macrofagen in het interstitium kan beschouwd worden als een homeostatisch proces waardoor het aantal monocyten/macrofagen gedeeltelijk onder controle wordt gehouden tijdens de massale influx van nieuwe monocyten.

In hoofdstuk 4 werd de interactie tussen PRRSV en LPS onderzocht. Het klinisch verloop bij PRRSV-LPS blootgestelde dieren en het effect van de frequentie van LPS toedieningen en het tijdsinterval daartussen werden nagegaan. Tevens werd onderzocht of de virulentie van de PRRSV stam de interactie beïnvloedt.

In deel 4.1. werd onderzocht of een PRRSV infectie de longen vatbaar maakt voor het ontstaan van ademhalingsstoornissen wanneer deze blootgesteld worden aan *Escherichia coli* LPS. Tevens werden een aantal cellulaire en virologische aspecten van de longen van deze dieren onderzocht. Conventionele biggen werden op 5 weken leeftijd intratracheaal geïnoculeerd met Lelystad virus en 5 dagen later werden ze één- of tweemaal intratracheaal geïnoculeerd met LPS. Enkelvoudig PRRSV- en LPS-geïnoculeerde dieren werden ingesloten als controles. Na de LPS toedieningen werden de dieren klinisch opgevolgd voor zowel algemene (koorts en depressie) als respiratoire (tachypnee, flankenslag en dyspnee) symptomen. Een aantal dieren werd na de tweede LPS toediening geëuthanaseerd voor onderzoek van de broncho-alveolaire cellen en virologisch onderzoek van de longen. PRRSV-LPS blootgestelde dieren vertoonden uitgesproken ademhalingssymptomen, gekenmerkt door tachypnee, flankenslag en dyspnee. De PRRSV en LPS controledieren vertoonden daarentegen geen of slechts milde ademhalingsproblemen. De meeste PRRSV en LPS controledieren vertoonden

weliswaar wel milde koorts, maar depressie werd niet waargenomen. De PRRSV-LPS blootgestelde dieren, daarentegen, vertoonden hoge koorts en een sterk uitgesproken depressie. Het effect van een tweede LPS toediening was afhankelijk van het tijdsinterval tussen de twee LPS toedieningen. Bij de LPS controledieren veroorzaakte een tweede LPS toediening, 24 uren na de eerste, minder kliniek dan de eerste. Een tweede LPS toediening, 3 uren na de eerste, zorgde daarentegen voor een uitgesproken verergering van de klinische symptomen. Het effect van een tweede LPS toediening bij PRRSV-geïnfecteerde biggen kon moeilijk beoordeeld worden, aangezien de dieren nog niet hersteld waren op het ogenblik van de tweede LPS toediening, 3 of 24 uren na de eerste. De infiltratie van neutrofielen in de longen tengevolge van de LPS toediening was gelijkaardig bij niet-geïnfecteerde en PRRSV-geïnfecteerde dieren. De virustiters in de longen waren gelijkaardig bij PRRSV-geïnfecteerde dieren, onafhankelijk van het feit of ze al dan niet aan LPS blootgesteld waren geweest. De ademhalingssymptomen na de PRRSV-LPS blootstelling konden dus niet verklaard worden door de graad van virusvermeerdering of door ontstekingsverschijnselen in de longen. Uit deze studie kan besloten worden dat er een duidelijk synergisme bestaat tussen PRRSV en LPS in de inductie van ademhalingssymptomen. Het klinisch synergisme werd waargenomen in 87% van de dieren en was sterk reproduceerbaar.

In deel 4.2. werd onderzocht of er eveneens een synergisme bestaat tussen verzwakte PRRSV stammen en LPS. Daartoe werden twee experimenten uitgevoerd. In beide experimenten werden conventionele biggen op 5 weken leeftijd intratracheaal geïnoculeerd met een levend verzwakt PRRSV vaccin, gebaseerd op enerzijds een Europees serotype of een Amerikaans serotype. Lelystad virus-geïnoculeerde en niet-geïnoculeerde dieren werden ingesloten als controles. Vijf dagen na de virusinoculatie werden de dieren intratracheaal geïnoculeerd met *Escherichia coli* LPS. De dieren werden om de 2 uren klinisch opgevolgd vanaf 0 tot en met 12 uren na de LPS toediening. Net als in deel 4.1. werden de dieren opgevolgd voor zowel algemene (koorts en depressie) als voor respiratoire (tachypnee, flankenslag en dyspnee) symptomen. Alle dieren uit het tweede experiment werden 12 uren na de LPS toediening geëuthanaseerd voor virologisch onderzoek van het longinterstitium en de broncho-alveolaire ruimten (virustitratie en kwantificatie van het aantal geïnfecteerde cellen). De LPS controledieren vertoonden lichte tot matige ademhalingssymptomen. Lelystad virus-LPS blootgestelde dieren, daarentegen, ontwikkelden uitgesproken ademhalingssymptomen na de LPS blootstelling. De ademhalingssymptomen van vaccinvirus-LPS blootgestelde dieren

waren minder uitgesproken en van kortere duur dan deze van de Lelystad virus-LPS controledieren. Deze verschillen kunnen eventueel verklaard worden door verschillen in het patroon en de graad van virusvermeerdering in de longen. De gemiddelde virustiters en het gemiddeld aantal geïnfecteerde cellen waren immers duidelijk lager bij de vaccinvirus-LPS blootgestelde dieren dan bij de Lelystad virus-LPS blootgestelde dieren. Viraal antigeen-positieve haardjes, bestaande uit groepjes van drie of meer geïnfecteerde cellen en celafval, werden bovendien uitsluitend waargenomen in het interstitium van de Lelystad virus-geïnoculeerde dieren. Deze studie toont aan dat er een synergisme bestaat tussen verzwakte PRRSV stammen en LPS, maar de klinische symptomen zijn minder uitgesproken dan met de virulente Lelystad stam.

In hoofdstuk 5 werd, met het oog op de preventie van de ademhalingsproblematiek bij varkens, aandacht besteed aan de virologische en klinische bescherming opgewekt door levend verzwakte PRRSV vaccins. De virologische bescherming werd nagegaan ter hoogte van de longen, het belangrijkste doelwitorgaan voor PRRSV. Naar ons weten is dit de eerste studie die de graad van virologische bescherming nagaat ter hoogte van de longen van PRRSV-gevaccineerde dieren. Voor de evaluatie van de klinische werkzaamheid werd gebruik gemaakt van het PRRSV-LPS model. Twee experimenten werden uitgevoerd. In beide experimenten werden conventionele dieren op zes weken leeftijd intramusculair geïmmuniseerd met een levend verzwakt PRRSV vaccin, gebaseerd op enerzijds een Europees serotype of een Amerikaans serotype. Niet-geïmmuniseerde dieren en dieren, intramusculair geïmmuniseerd met de virulente Lelystad stam (infectie-immune dieren), werden ingesloten als controles. De dieren werden zes weken na de immunisatie onderworpen aan een intratracheale (exp. 1) of intranasale (exp. 2) challenge met de Europese Lelystad stam. Drie en zes dagen na de challenge werden de dieren intratracheaal geïnoculeerd met *Escherichia coli* LPS. Na de LPS toediening werden de dieren klinisch opgevolgd. Vier en zeven dagen na de challenge werden dieren geëuthanaseerd voor virologisch onderzoek van de longen. De virologische bescherming ter hoogte van de longen was onvolledig bij beide gevaccineerde groepen, maar de vermeerdering van het challengevirus was meer gereduceerd bij de dieren geïmmuniseerd met de verzwakte Europese stam. De genetische en antigene verschillen tussen Europese en Amerikaanse PRRSV isolaten bepalen dus de graad van virologische bescherming ter hoogte van de longen. Ook de infectie-immune dieren vertoonden geen volledige virologische bescherming ter hoogte van de longen. Bij de infectie-immune dieren werden geen of slechts lichte

ademhalingssymptomen waargenomen na de LPS toediening. Niet-geïmmuniseerde challenge controledieren, daarentegen, vertoonden uitgesproken ademhalingssymptomen na de LPS toediening. Het klinisch verloop na de LPS toediening bij de gevaccineerde groepen was sterk variabel binnen en tussen beide experimenten. Géén van de dieren geïmmuniseerd met de verzwakte Europese stam ontwikkelden ademhalingssymptomen in experiment 1, terwijl in experiment 2 alle dieren uitgesproken ademhalingssymptomen vertoonden. Alle dieren geïmmuniseerd met de verzwakte Amerikaanse stam vertoonden ernstige ademhalingssymptomen in het eerste experiment, terwijl de ademhalingssymptomen minder uitgesproken waren bij de dieren uit het tweede experiment. We waren dus niet in staat om bij de gevaccineerde dieren reproduceerbare klinische resultaten te bekomen en het was dus onmogelijk om conclusies te trekken aangaande de klinische bescherming opgewekt door levend verzwakte PRRSV vaccins.

Als algemene conclusie kan gesteld worden dat een enkelvoudige PRRSV infectie duidelijke cellulaire veranderingen veroorzaakt ter hoogte van de longen en desalniettemin leidt dit niet tot ademhalingsproblemen. De pathogenese studies toonden ook aan dat apoptose wellicht een belangrijke rol speelt bij deze cellulaire veranderingen, hetzij als oorzaak, hetzij als gevolg.

Met de PRRSV-LPS combinatie werd duidelijk aangetoond dat een PRRSV infectie in staat is om de longen vatbaar te maken voor ademhalingsproblemen wanneer deze vervolgens blootgesteld worden aan LPS. In tegenstelling met vroeger uitgevoerde dubbelinfecties met PRRSV en andere virussen of bacteriën, is de PRRSV-LPS combinatie bovendien duidelijk reproduceerbaar. Deze combinatie kan dan ook beschouwd worden als een goed model om de pathogenese van multifactoriële ademhalingsproblemen te bestuderen.

Onze vaccinatie/challenge experimenten hebben duidelijk aangetoond dat de virologische bescherming in de longen van gevaccineerde dieren slechts partieel is. Het kan bijgevolg niet uitgesloten worden dat de vermeerdering van challengevirus in de longen van gevaccineerde dieren deze dieren nog kan vatbaar maken voor multifactoriële ademhalingsproblemen.

Curriculum vitae

PERSONALIA

Geoffrey Labarque werd op 28 september 1973 geboren te Kortrijk. In 1991 beëindigde hij zijn secundaire opleiding aan het Sint-Amandscollege te Kortrijk en behaalde hij het getuigschrift van hoger secundair onderwijs (Klassieke talen Latijn met wiskunde). In 1997 werd het diploma dierenarts behaald aan de faculteit diergeneeskunde van de universiteit Gent. Vanaf juli 1997 tot en met april 1998 was hij tewerkgesteld in het laboratorium voor virologie op het project "Gecombineerde intranasale – intramusculaire vaccinatie voor eliminatie van het Aujeszkyvirus in de Vlaamse varkenspopulatie in het kader van het eradikatieplan" gefinancierd door het Ministerie van Middenstand en Landbouw. In november 1998 kreeg hij een onderzoeksmandaat van het Bijzonder Onderzoeksfonds (BOF) van de universiteit Gent in het laboratorium voor virologie voor het project "Studie van de interactie tussen longmacrofagen en het porcien arterivirus bij het varken". In juni 2002 behaalde hij het getuigschrift voor zijn doctoraatsopleiding.

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